Section 4: Pathogen and Disease Management

SYMBIOTIC CONTROL OF PIERCE'S DISEASE: TESTING REAGENTS AGAINST *XYLELLA FASTIDIOSA*

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ABSTRACT
Pierce's disease (PD) is caused by a xylem limited gram-negative *Xylella fastidiosa* (*Xf*) bacterium. Various species of sharpshooters, including the important glassy-winged sharpshooter (GWSS), transmit *Xf*. Currently, there is no cure for PD. Paratransgenesis is a new tool for the management of PD. Acquisition efficiency of GWSS to acquire *Xf* is about 80% when tested with Real-Time PCR. Results of selected phage antibody specific to *Xf* PD-strain to disrupt the pathogen are underway.

INTRODUCTION
Strains of *Xylella fastidiosa (Xf)*, a gram-negative bacterium, cause a number of important plant diseases including Pierce's disease (PD) in grapevine, citrus variegated chlorosis (CVC) in citrus, phoney peach disease, periwinkle wilt, and leaf scorch disease in plum, elm, maple, sycamore, and coffee (Hopkins 1989).

The principal vector for the transmission of *X. fastidiosia* is the glassy-winged sharpshooter (GWSS) (*Homalodisca coagulata*). The pathogen attaches to the cibarium and precibarium of sharpshooters by means of an extracellular matrix (ECM) and is transmitted from infected plants to healthy plants when the sharpshooters feed (Brlansky et al. 1983).

Symbiotic control identifies a symbiont that is genetically modified to produce a gene product that inhibits transmission of a pathogen. Recent examples of symbiotic control are the control of Chagas' disease caused by *Trypanosoma cruzi* and transmitted by the Triatomid bug *Rhodnius prolixus* (Durvasula et al. 1997), the prevention of Colitis in mammals (Beninati et al. 2000, Steidler et al. 2001), and to interfere with HIV transmission (Chang et al. 2003).

This approach is being developed for the management of PD. *Alcaligenes xylosoxidans* subsp. *denitrificans* (*Axd*) was chosen for genetic modification to deliver an anti-*Xylella* product. *Axd* is appropriate because it shares the same niche as *Xf* in the foregut of the GWSS and cycles well between the insect and plant system. Also, this bacterium has been described as a non-pathogenic soil-borne microbe and a non-pathogenic endophyte (Meade et al. 2001).

Single chain antibody (scFV S1), which is expressed on the surface of a M13 bacteriophage, has been selected against *Xf* PD-strains by using a panning technique. S1 is supposed to bind to the surface of a *Xf* PD-strain. Currently we are testing S1 in an *in vitro* insect-plant-pathogen system.

OBJECTIVES
1. Test the acquisition of *Xf* by GWSS feeding on infected *Vinca major*.
2. Test the efficiency of S1 to inhibit *Xf* transmission on *V. major*.

RESULTS
Field collected GWSS from a citrus orchard were put into an artificial feeding system (AFS) to acquire S1. Afterwards the GWSS were allowed an acquisition access period (AAP) on a *Xf* PD-strain infected *V. major* for 48 hours. Then, these sharpshooters were transferred onto clean test *V. major* plants and allowed an inoculation access period (IAP) of 48 hours. After 6 weeks these test plants were tested for *Xf* colonization by Real-Time PCR (rt-PCR). Negative controls were an anti-BSA phage and PBS. Each of the three treatment groups was mixed with 0.2% dextrose in a 1:4 ratio, respectively. The AFS consists of multiple plastic vials each with a vinyl tube both closed with wrapped parafilm and filled with the appropriate above said solution.

In another set of experiments the field collected GWSS were allowed an AAP of 48 hours on the *Xf* PD-strain infected *V. major*. Then these GWSS were transferred to the AFS to acquire S1, anti-BSA phage, and PBS solution for 48 hours.

Thereafter these GWSS were allowed an IAP on clean *V. major* for 48 hours. And then these plants were tested for *Xf* colonization via rt-PCR after 6 weeks.

In both sets of experiments the transmission of *Xf* by GWSS was tested by allowing the sharpshooters to feed first on the *Xf* PD-strain infected *V. major* for 48 hours. Then these test insects were transferred onto clean test plants to feed for 48 hours. The results of the experiments are pending.

Samples of the GWSS that fed on the *Xf* PD-strain infected plant for 48 hours were taken and then their heads were tested for the presence of *Xf* via rt-PCR. Eighty percent (range 70-100%) of GWSS heads shows the presence of *Xf*. The field collected GWSS were also tested for the presence of *Xf* via rt-PCR. Only 0-10% (mean = 5%) of the field collected GWSS were found to be infected with *Xf*.

CONCLUSION

An effective AFS has been developed to allow the GWSS to acquire S1. *V. major* was selected as the model plant for our insect-plant-pathogen system to test S1. Eighty percent of the GWSSs acquire *Xf* after 48 hours of AAP. Experiments on the disruption of *Xf* by S1 are ongoing.

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EXPLOITING XYLELLA FASTIDIOSA PROTEINS FOR PIERCE'S DISEASE CONTROL

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ABSTRACT

The principal objective of this project is to construct and express in test plants, and then in grapevine rootstock, a protein or protein chimera capable of inactivating *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease (PD) of grapevine. Prior results from this project identified MopB as a, or possibly the, major outer membrane protein of *Xf*. We have shown that MopB is accessible on the *Xf* cell exterior and is a member of the OmpA family of outer membrane proteins of Gram-negative bacteria. The abundance of MopB in *Xf* cell extracts, known packing density of OmpA in a crystal, and *Xf* cell dimensions allowed us to estimate that MopB probably accounts for at least 10% of the *Xf* cell exterior. Thus, MopB is a highly suitable target for inactivation of *Xf* cells. Previous results suggested that some portion of the intact MopB gene from *Xf* is sickening to *E. coli*. Nevertheless, two *E. coli* strains were generated by substitution into the endogenous *OmpA* gene, one expressing mature MopB and the other a MopB-OmpA chimera with the amino-terminal half from MopB. Cells of both strains display MopB antigen on their surface, though accumulation is to a level much lower than MopB achieves in *Xf*. The strains are immune to bacteriophage K3, for which OmpA is the receptor. We modified and randomly mutated the OmpA-binding gp38 adhesin protein of bacteriophage K3 and will use *Xf* cells and the MopB-surface *E. coli* strains described above to select bacteriophage K3 variants that use MopB as the receptor. The selected gp38 gene will form the core of an anti-*Xf* protein. A readily transformed and regenerated tobacco line, SR-1, was identified as being susceptible to *Xf* and producing PD-like symptoms and cytology. SR-1 will be a used to test anti-*Xf* proteins and optimize constructions for grapevine transformation. High level expression of a fragment of MopB has been achieved and the same technology will be used to obtain sufficient quantities of MopB to complete its biological characterization.

INTRODUCTION

Resistant grapevine cultivars present the best approach to long term, effective, economical and sustainable control of Pierce's disease (PD). This project has developed data showing that the OmpA class protein MopB of *Xylella fastidiosa* (*Xf*) is a major outer membrane protein of the bacterium. The demonstrated accessibility of parts of the MopB molecule on the cell surface and its abundance identify MopB as a high priority potential target for inactivation of the *Xf* cell or interference with the *Xf* infection cycle. As background, results from our prior research are summarized below (Bruening et al. 2005):

a. *Xf* cells, fresh or heat-killed, when pressure-infiltrated into *Chenopodium quinoa* leaves, induce within two days chlorosis (chloroplast bleaching) that is limited to the infiltrated area of the leaf (CqC activity).
b. The CqC activity is protease sensitive and was associated with a gel electrophoresis band that was found, by mass spectrometry, to contain predominantly the putative (OmpA class) *Xf* outer membrane protein MopB.
c. The mature, 38.5K MopB protein was found to result from the release of a 22 amino acid leader peptide. The bulk of mature MopB molecules have a pyroglutaminyl amino end.
d. MopB was partially purified in soluble form using sodium dodecyl sulfate (SDS) solutions but reducing, at the last step, SDS to very low levels.
e. Application of anti-MopB antibody demonstrated that MopB is accessible on the *Xf* cell exterior and appears to be evenly distributed over the *Xf* cell surface.
f. MopB is an abundant protein of *Xf* and may be the major outer membrane protein of the bacterium.
g. *E. coli* did not tolerate plasmid constructions bearing the entire MopB gene from *Xf*. However, placing the MopB open reading frame under control of a bacteriophage-derived promoter allowed the production of low amounts of MopB in *E. coli.*
h. Both purified MopB and MopB still embedded in *Xf* cells showed a strong propensity to associate tightly with porous materials of a variety of chemical types. This result is reminiscent of the observed association of a *Pseudomonas fluorescens* OmpA protein, OprF, with root surfaces (De Mot and Vanderleyden 1991, Deflaun et al. 1994), MopB may be involved in the association of *Xf* cells with the interior of xylem elements in the inoculated plants.

Our principal objective is to construct and express in test plants, and then in grapevine rootstock, a MopB-binding protein (MBP) or protein chimera capable of inactivating *Xf*. We expect that a construction of suitable design will confer, on the

grapevine scion, resistance to *Xf*. If rootstock expression does not confer scion resistance to *Xf*, we will turn to transformation of the scion. To obtain a high affinity MBP, we are modifying a protein of a T2-like bacteriophage: the tail fiber adhesion gp38 (Riede et al. 1987). The gp38 protein of bacteriophages K3, M1 and OX2 recognizes and binds to *E. coli* OmpA, the receptor for bacteriophage infection. Based on the rapidity and irreversibility of bacteriophage association with *E. coli*, gp38 likely binds very tightly to OmpA. Bacteriophage mutants with "shifted allegiance" away from OmpA and to other *E. coli* surface proteins were selected using *E. coli* mutants with altered or missing OmpA. In one instance, the new receptor was a polysaccharide rather than an outer membrane protein (Drexler et al. 1991). The mutations controlling affinity for the new receptor mapped to four polypeptide loops of gp38 (Drexler et al. 1989). We believe a mutated gp38 could have a high affinity for MopB. OX2 apparently has been lost. M1 has been more readily adapted than has K3 to new receptors (Henning and Hashemolhosseini 1994). We obtained inocula of bacteriophage K3 and bacteriophage M1 from a former postdoctoral associate in the laboratory of Ulf Henning (deceased) in Germany.

The predominant conformation of a typical OmpA protein, as it resides in the outer membrane of *E. coli*, almost certainly has the polypeptide chain composed of amino acid residues 1-171 inserted into the outer membrane with 8 trans-membrane segments and four external loops (Pautsch and Schulz 1998, Singh et al. 2003). We have cast MopB into a similar conformation based on the crystallographic structure of OmpA (Pautsch and Schulz 1998) and computer predictions of folding for OmpA and MopB and have initiated research aimed at converting gp38 from a OmpA-binding protein to a MBP. That is, our initial aim is to select a version of gp38 that has been modified in its receptor-binding four loops to recognize and adhere tightly to the cell-external four loops of MopB.

OBJECTIVES

The goal of this project is to generate *Xf*-resistant grapevine rootstock and plants based on expression of a MBP.

Specific objectives:

1. Discover or develop low molecular weight MBPs with high affinity for portions of the MopB protein that are displayed on the *Xf* cell exterior.
2. Test MopB-binding proteins for their ability to coat *Xf* cells, for possible bactericidal activity, and for interference with disease initiation following inoculation of grape with *Xf*.
3. In collaboration with the Gupta laboratory, develop gene constructions for chimeric proteins designed to bind tightly to and inactivate *Xf* cells; express and test the chimeric proteins for their effects on *Xf* cells in culture.
4. In collaboration with the Dandekar laboratory, prepare transgenic grape expressing the candidate anti-*Xf* proteins; test the transgenic plants for resistance to infection by *Xf*.

RESULTS

Under Objective 1 (discover MBPs)

Expression of MopB on the E. coli cell surface

Obtaining *E. coli* cells that express MopB sequences and display MopB surface polypeptide loops, as is characteristic of MopB in *Xf*, is central to our selection procedure for MBPs. As was reported in the previous period, we created an *E. coli* strain that was designed to display MopB sequences on the cell exterior but to otherwise be compatible with expression in *E. coli*. Using a gene-replacement approach, a recombinational event replaced the amino terminal region, residues 1-171, of the chromosomal *OmpA* gene of *E. coli* with the corresponding region of MopB. This construction retains the OmpA signal peptide and the OmpA carboxyl half of the molecule, which includes the trans-periplasmic space sequences and the sequence that is inserted into the peptidoglycan layer. The replacement was confirmed by sequence analysis after PCR amplification of the chimeric gene region from chromosomal DNA. A similar approach has now produced an OmpA replacement which was designed to generate the entire mature MopB molecule. Cells of the new *E. coli* strains were found to be entirely resistant to bacteriophage K3 and to be agglutinated by beads displaying anti-MopB IgG, as expected. Immunoblots were prepared after SDS-PAGE of *E. coli* hot-SDS cell extracts for the two strains. Results (not shown) revealed the accumulation of MopB-like proteins of the expected mobility. However, the MopB-immunoreactive material from the *E. coli* strains amounted to no more than a few percent of the signal observed for similar amounts (total protein) of *Xf* cells. Expression of mature MopB and MopB-OmpA chimera proteins appears to be below the level of OmpA accumulation in wildtype *E. coli*. We suspect that there is a codon usage problem for the synthesis of MopB and MopB-OmpA chimera in *E. coli* and are taking steps to introduce the cognate tRNAs.

Modification of gp38 for adhesion to MopB.

We attempted to find bacteriophage K3 variants capable of infecting MopB-OmpA chimera-bearing *E. coli* cells. The cells were exposed to 10^{10} plaque forming units of untreated bacteriophage K3 and to bacteriophage K3 populations that had been treated with the mutagen hydroxylamine or that had been increased in cells exposed to the *in vivo* mutagen 2-aminopurine. No infecting K3 variant was found. Henning and Hashemolhosseini (1994) report that bacteriophage M1 is more suited than K3 to adaptation to receptors other than OmpA. We obtained bacteriophage M1 inoculum, but found it to behave similarly to K3 in our tests. PCR amplification of a gp38 sequence from the "M1" DNA revealed the sequence of K3 gp38, suggesting that bacteriophage M1, like bacteriophage OX2, may no longer be available. We are in the process of creating a library of mutated gp38 sequences based on the published M1 gp38 sequence. These will be introduced into bacterophage K3 to create

a library of mutants for selection of MopB-binding gp38. Selection will use *Xf* cells as well as the MopB-surface *E. coli* strains.

MopB and the CqC assay

Cell suspensions from the chimeric MopB-OmpA and mature MopB *E. coli* strains were pressure infiltrated into *C. quinoa* leaves. Both cell suspensions, as well as wildtype *E. coli* cell suspensions, behaved similarly. A CqC reaction was observed at the higher cell suspension densities, and the reaction was similar for all three suspensions. That is, *E. coli* appears to have an endogenous CqC-like activity. Given the demonstrated low accumulation of MopB-OmpA chimeric protein and MopB in the *E. coli* strains, CqC activity from the expressed proteins, if any, most likely was overshadowed by the endogenous CqC-like activity of *E. coli*.

We are preparing constructions for expression of intact MopB and specific MopB fragments using the high level expression (Dubendorff and Studier 1991) pET160 plasmid system. The first to be completed produced the carboxyl half of MopB, as indicated in Figure 1. The CqC assay of the purified protein preparation (analyzed at Figure 1, lane 5) failed to induce chlorosis when infiltrated into *C. quinoa* leaves, whereas control preparations of *Xf* cells induced the usual CqC reaction, suggesting that CqC activity does not reside in the carboxyl half of MopB alone.

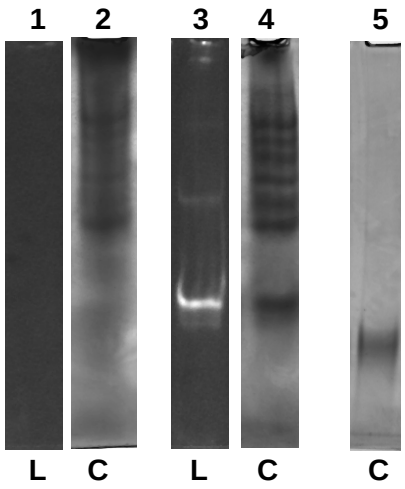

Figure 1. Production of microgram amounts of the carboxyl half of *Xf* outer membrane protein MopB in transformed *E. coli*. Sequence encoding the carboxyl half of the mature MopB protein was inserted into a pET160 plasmid vector on the 3'-side of tetracysteine- and hexahistidine-encoding sequences and under control of a bacteriophage T7 promoter and *lac* operator sequence. The construction was transformed into an *E. coli* strain that lacks a bacteriophage T7 RNA polymerase gene for characterization and propagation of the clone. This approach is intended to prevent even basal level expression of the insert sequences. For high level expression, purified plasmid is transformed into BL21 Star *E. coli* cells, which encode T7 RNA polymerase under control of a *lacUV5* promoter. Cultures were grown up directly without isolation of individual colonies and were exposed (lanes 3-5) or not exposed (lanes 1, 2) to the gratuitous inducer IPTG. Protein extracts were incubated with the tetracysteine-binding fluorescent lumio reagent prior to electrophoresis through 12% polyacrylamide in SDS. Detection was by lumio fluorescence (lanes 1 and 3, L) or by staining of the gel with coomassie brilliant blue (lanes 2, 4, and 5, C). Lanes 1-4 are from one gel. Material for lane 5 was purified from lane 3 material by nickel column chromatography in urea solution.

Under Objective 4 (transgenic plant expression of anti-Xf protein)

Test bed for analysis of constructions designed to express anti-Xf protein

To facilitate our goal of creating grape rootstock that can confer resistance to *Xf* on its grafted scion, we developed a plant model system for rapid transformation with anti-*Xf* constructions and rapid testing for phenotype (Francis et al. 2005), compared to grapevine. Constructions discovered to have promising anti-*Xf* activity will be used to transform a grapevine rootstock line. We have demonstrated that tobacco (*Nicotiana tabacum*) line SR-1, which is routinely transformed and regenerated at the UC Davis College of Agricultural and Environmental Sciences Plant Transformation Facility, is readily infected by needle inoculation into the petiole axil or stem. *Xf* was recovered from the petiole above inoculation points, whereas no bacteria were recovered from water-inoculated controls. Symptoms developed (Figure 2A) and *Xf* accumulated, as indicated by ELISA, quantitative PCR, and clogging of xylem vesicles (Figure 2B), providing unequivocal evidence of infection. Others have succeeded in infecting N. tabacum strains with *Xf* (Lopes et al. 2000, Alves et al. 2003). The symptoms we observe appear to be more dramatic than those reported. *Xf* isolated from SR-1 tobacco caused typical PD symptoms following artificial inoculation to grapevines (Figure 2C).

CONCLUSIONS

The goal of this project is to create genes encoding anti-*Xf* proteins for transformation of grape rootstock and protection of the grafted scion against PD. *E. coli* strains were created that display on the cell exterior portions of a *Xf* major outer membrane protein, MopB. These strains are expected to be suitable hosts for a bacteriophage that will accept a displayed portion of MopB as a receptor. A synthetic bacteriophage gp38 adhesin gene has been randomly mutated and will be incorporated into a population of bacteriophage K3 to produce a library from which bacteriophage strains that use MopB as a receptor will be selected. The selected gp38 gene will form the core of an anti-*Xf* protein. High level expression of a fragment of MopB has been achieved and will be applied to full length MopB to complete its biological characterization. A

readily transformed and regenerated tobacco line, SR-1, has been identified as a suitable platform for testing and optimizing anti-*Xf* protein gene constructions.

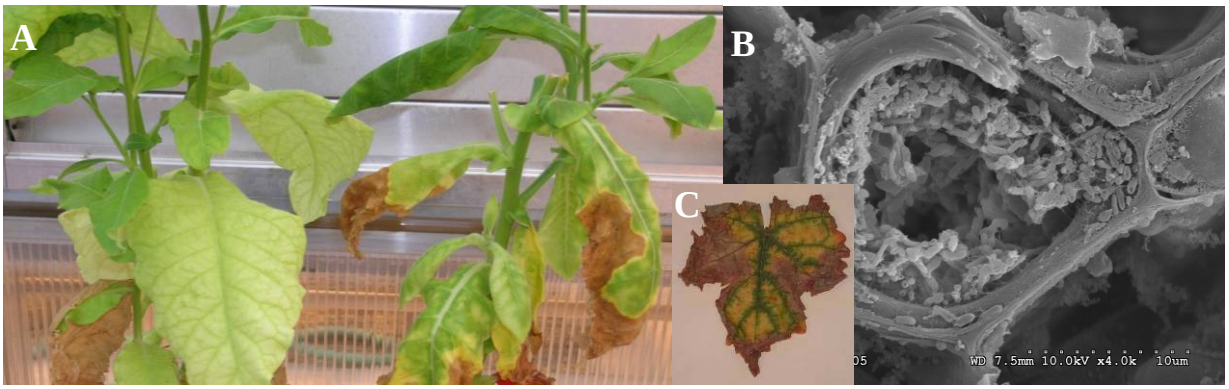

Figure 2. SR-1 tobacco as a host for *Xf*. A. Water-infiltrated (left) and *Xf*-inoculated plants 3 months after inoculation at the 6 leaf stage. Although leaves of control plants developed senescence, none developed the downward curvature, cupping and tip- and margin-necrosis with chlorotic halo that are characteristic of the *Xf*-inoculated plants. 4/4 leaves from two control plants were negative for *Xf* by ELISA and PCR. Extracts of 7/7 leaves from three *Xf*-inoculated plants generated ELISA signals averaging 4x the control level; quantitative PCR signals exceeded the threshold product accumulation at 19-31 cycles. B. Electron microscopy of SR-1 petiole sections at 10-12 nodes above the inoculated leaf revealing bacterial cells occluding a xylem element. C. Sap from Temecula-1 *Xf*-inoculated, symptomatic SR-1 tobacco was inoculated to grapevine cuttings, resulting in typical PD symptoms and accumulation of *Xf*.

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SIDEROPHORE PRODUCTION OF *ALCALIGENES XYLOSOXIDANS DENITRIFICANS* AND POTENTIAL BIOLOGICAL CONTROL AGAINST *XYLELLA FASTIDIOSA*

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ABSTRACT
Our overall objective is to determine siderophores produced from *Alcaligenes xylosoxidans denitrificans* (*Axd*), isolated from glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* Say (Hemiptera: Cicadellidae) (Bextine et al. 2004), and to investigate the ability of *Xylella fastidiosa* (*Xf*) to obtain iron from siderophores produced by *Axd*. We are also interested in the effect of siderophores as a potential biological control against *Xf*.

INTRODUCTION
Most bacteria must acquire iron by competing with environmental chelation. One mechanism for bacterial iron acquisition utilizes siderophores (Kline et al. 2000). Siderophores are small molecules that bind extracellular iron with high affinity (Neilands 1995). The presence of coding genes for iron uptake membrane receptors in *Xf* (Simpson et al. 2000) suggest that *Xf* biosynthesize and uptake siderophores (Silva-Stenico et al. 2005).

OBJECTIVES
1. Determine if *Axd* produces siderophores that *Xf* can binds.
2. Investigate the interaction between *Axd* and *Xf* in iron-restricted environment.

RESULTS AND CONCLUSIONS
By using CAS-agar assay (Schwyn and Neilands 1987), the difference strains of *Axd* have been tested for siderophores production. This is due to the difficulty of promoting production of siderophores suggesting that siderophores are crucial for biosynthesis. We are investigating a potential biological control of siderophores against *Xf*.

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Additional Notes: All of the field tests were conducted under a permit from the Environmental Protection Agency (TERA R-03-01). A report of the tests was submitted to EPA and the sponsors.

EFFECTS OF GROUP, CULTIVAR, AND CLIMATE ON THE ESTABLISHMENT AND PERSISTENCE OF *XYLELLA FASTIDIOSA* INFECTIONS CAUSING ALMOND LEAF SCORCH

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ABSTRACT
Almonds are one of the most widely-grown crops that can host *Xylella fastidiosa (Xf)*, so it is useful to assess the potential for almonds to serve as a source of *Xf* infections in grapes, and to explain why *Xf* dispersal between almond orchards and vineyards is uncommon. We are comparing infection establishment and survival at two field sites and in a controlled test with potted plants, varying three factors that may influence almond leaf scorch in almonds: cultivar, genetics of the pathogenic bacteria, and winter severity. In spring and summer 2005, we inoculated grape type and two almond types of *Xf* into highly susceptible 'Peerless' and less-susceptible 'Butte' almond trees. After vector inoculation, *Xf* must survive multiple winters in an almond tree to reach sufficient populations for sharpshooter acquisition and economic impact disease levels. Therefore, field sites were selected with moderate and severe winter temperatures. We also initiated a controlled dormancy test with potted plants and cold storage rooms at Kearny Agricultural Center, Parlier, California. Almond trees in the field were inoculated with buffer or *Xf* belonging to the grape type or two almond types, and will be held at different chill temperatures for varying lengths of time.

INTRODUCTION
Because almonds are one of the most widely-grown crops that can host *Xf* in the Central Valley, they might serve as a source of *Xf* infections in grapes, although for unknown reasons *Xf* dispersal between almond orchards and vineyards is uncommon (A. Purcell – *unpublished data*). Almond leaf scorch (ALS) is caused when *Xf* multiplies extensively within the xylem of infected trees, eventually severely limiting nut production (Davis et al. 1980). The disease was first formally described in 1974, though outbreaks occurred in Los Angeles and Contra Costa counties in the 1950's (Moller et al. 1974). Symptoms of ALS are similar to Pierce's disease (PD) in grapes and include leaves with marginal necrosis and chlorosis and the lack of terminal growth. Initial infections spread slowly and often occur only on one branch, but after a few years are easily visible on the entire tree (Almeida and Purcell 2003c), reducing almond productivity (Mircetich et al. 1976, Moller et al. 1974). In both grapes and almonds, *Xf* multiplies to high populations (1,000,000 bacteria per gram of plant tissue) and is acquired and transmitted by insect vectors (Almeida and Purcell 2003a, Almeida and Purcell 2003c, Purcell 1980a). In laboratory tests, *Xf* was transmitted to almonds by 5 species of xylem-feeding insects, including the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Almeida and Purcell 2003b, 2003c).

In previous studies, almond cultivars varied greatly in their susceptibility to ALS, with some developing extensive leaf scorch, and others showing little disease. *Xf* inoculations made from May through July had the best odds of surviving the following winter. (B. Kirkpatrick – *unpublished data*). We compared *Xf* infection establishment and survival in two cultivars, highly susceptible 'Peerless' and less-susceptible 'Butte,' both on Nemaguard rootstock.

The genetic type of *Xf* may also impact almond susceptibility, and will certainly impact formation of PD. Three genetic types of *Xf* have been identified from almond trees. One type was identical to *Xf* from PD-infected grapevines. The other two genetic types were unique to almonds (Hendson et al. 2001). The three types were distinguished by growth on selective media and DNA digestion with restriction enzymes (Almeida and Purcell 2003c). Recent cross-inoculation studies in the greenhouse showed that the genetic type influenced the ability of the bacteria to over winter in grapes or almonds, as almond types died in grapes and grape types died in almonds (Almeida and Purcell 2003c). For this reason, we also used different genetic types of *Xf* in our field trials.

After vector inoculation, *Xf* must survive multiple winters in an almond tree to reach sufficient populations for sharpshooter acquisition and economic impact disease levels. Growth chamber and field studies with grapevines showed that the degree of plant dormancy, as well as severe cold, affected the over winter survival of *Xf* (Feil and Purcell 2001, Purcell 1980b). To date, there is no information available on the effects of winter dormancy on *Xf* infections in almonds. Growth chamber and

field studies with grapevines showed that the degree of plant dormancy, as well as severe cold, affected the over winter survival of *Xf* infections. To date, there is no information available on winter dormancy effects on *Xf* infections in almonds.

We are comparing infection establishment and survival at two field sites and in a controlled test with potted plants, varying three factors that may influence almond leaf scorch in almonds: cultivar, genetics of the pathogenic bacteria, and winter severity. Therefore, field sites were selected with moderate and severe winter temperatures (Armstrong Farm at University of California, Davis, and Intermountain Research and Extension Center at Tulelake, California, respectively) in order to study treatment impact under different winter temperatures. A controlled dormancy severity test with potted plants and growth chambers was also started at Kearny Agricultural Center, near Parlier, California.

OBJECTIVES

1. Compare the establishment and multi-year persistence of *Xf* isolates belonging to three ALS genetic groups in almond cultivars with either low or high susceptibility to almond leaf scorch.
2. Compare effects of winter severity and the degree of plant dormancy on the infection rate, symptom severity, and titer of *Xf* in inoculated almonds.
3. Use collected data on almond leaf scorch development to determine if almond orchards may serve as a reservoir of *Xf*.

RESULTS

Field trials

One hundred bare-root almond trees, fifty of each cultivar, were planted in spring 2005 at two different field sites: Armstrong Farm at University of California, Davis, (hereafter referred to as UCD), and Intermountain Research and Extension Center, Tulelake, CA (hereafter referred to as IRC). Trees were planted in a complete randomized block design with a split plot (almond cultivars) in each block. There are ten replicates of each treatment combination (*Xf* isolate x almond cultivar). Trees are drip irrigated at UCD and sprinkler irrigated at IRC.

The almonds trees were inoculated with different genetic types of *Xf.* In our study, each tree was inoculated with one of five treatments: Fresno-ALS (isolated from almonds but genetically similar to *Xf* that causes PD in grapes; PD-*Xf*), Dixon (ALS-*Xf* type 1) and ALS 6 (ALS-*Xf* type 2), Medeiros (from grapes), or buffer control. All isolates of *Xf* were isolated from infected plants in Solano, Fresno, or San Joaquin Counties, and were pathogenic in recent greenhouse tests. Inoculations were done in early May (UCD) and early July (IRC) when the young shoots were at least 6 mm in diameter. Inoculum was prepared in the field from two week old cultures *Xf* grown on solid media. Each isolate was mechanically inoculated into 3 or 4 sites in 1 stem per plant by placing a 10 µL drop of bacteria suspended in sodium-citrate-phosphate buffer (approximately 10,000,000 bacteria/ mL). The drop was placed on a green, growing shoot and probed with a #2 insect pin until it was drawn into the stem. Inoculation sites were marked with permanent metal tags and paint.

Leaves immediately adjacent to the inoculation sites were tested for *Xf* in fall 2005 to see if inoculations were successful. The severity of infection was rated by the number of scorched leaves on the inoculated stem. Almond petioles from each tree were cultured to determine *Xf* infection and population. Subsequent strain identification of *Xf* was accomplished by re-streaking growing bacteria on two different artificial media, PD3 and PWG (Davis et al 1983, Davis et al 1980, Hill and Purcell 1995). All types of *Xf* grow on PWG, while ALS-*Xf* type 2 and PD types grow on PD3 as well. ALS-*Xf* type 1 does not (Almeida and Purcell 2003c). To separate ALS and PD isolates, polymerase chain reaction (PCR) was used to amplify DNA from the bacteria, and DNA was digested with *Rsa 1,* a restriction enzyme that cuts the DNA of ALS-*Xf* isolates into two pieces, but does not cut the DNA of PD-*Xf* (Almeida and Purcell 2003c). We will be able to know what infections over wintered by summer 2006, and compare infection establishment, bacterial titer, and rate of disease development in field-grown almond trees. Trees will also be evaluated for the presence of *Xf* in 2007 and 2008.

A preliminary screening found that almond leaf scorch symptoms were much more severe at the UCD site, especially in 'Peerless' trees, with an average of 4.6 scorched leaves per tree, compared to 0.8 in 'Butte.' Both cultivars at IRC had no scorched leaves, an average of 0.2 and 0.1 leaf per tree for 'Butte' and 'Peerless,' respectively. However, there was no difference in the proportion of infected trees at UCD (32 of 78 infected at UCD, 41 of 96 infected at IRC; Chi-square $P > 0.05$), nor in the median populations of *Xf* present in inoculated trees at UCD (6.2×10^6 CFU/g) or IRC (1.3×10^7; $\log_{10}$-transformed; $P = 0.26$). The difference in symptoms may have two explanations: i) trees at UCD were tested for *Xf* 3.5 mos after inoculation and had longer to develop symptoms, compared to trees at IRC, which were tested 2 months after inoculation; or ii) the infected trees were under more moisture stress at UCD, which led to the development of disease symptoms.

There were not large differences between infection percentage (41% of 'Butte,' 38% of 'Peerless'; Chi-Square $P>0.05$), or *Xf* population (2×10^6CFU/g for 'Peerless' and 9×10^6CFU/g for 'Butte'; $\log_{10}$-transformed; $P = 0.11$) for the two cultivars. 'Peerless' had much fewer scorched leaves than 'Butte' at UCD, but not at IRC, as discussed in the previous paragraph.

One significant difference was the infection percentage of the various isolates, as grape strain *Xf* was more frequently recovered from inoculated trees than either almond strain. Fresno and Medeiros were recovered from 64 and 77% of trees, respectively, whereas ALS6 and Dixon were recovered from 27 and 28% of trees. Leaf scorch symptoms were more severe

in trees inoculated with grape-type isolates Fresno and Medeiros (an avg. of 2.8 and 3.2 scorched leaves/tree), compared to almond isolates Dixon and ALS6 (0.3 and 0.9 scorched leaves/tree), and background leaf scorch in buffer-inoculated trees (0.1/ tree).

Bacterial populations in trees infected with grape and almond isolates were similar, even though infection percentage and symptom severity was greater in grape isolates of *Xf*. Median populations of *Xf* in infected trees were: 6.2 x 10^6 CFU/g (ALS6), 2.8 x 10^6 CFU/g (Dixon), 5.5 x 10^6 CFU/g (Fresno), 2.4 x 10^7 CFU/g (Medeiros), and 0 CFU/g (buffer). Bacterial populations were high even in only a few trees in the treatment were infected with *Xf*, as in ALS6 inoculated plants at UCD. In the future, ArcSin transformation may be necessary with infection data, and log10 transformation may be necessary to analyze population data. We will use ANOVA where applicable to detect differences in infection percentage and bacterial populations between cultivars and bacterial isolates.

Glasshouse and Growth Chamber trial

An additional experiment was initiated to examine the effect of over wintering temperature in the survival of *Xf* infections in controlled environments. We inoculated 155 potted two-year-old 'Peerless' almond trees in spring 2005. One hundred twenty five trees were inoculated with the ALS 6 isolate of *Xf* and 30 with buffer alone, in the same manner as for the field plots. Trees were kept in the greenhouse at Kearny Agricultural Center (Parlier, CA) and were tested for infection in fall 2005. Only trees positive for *Xf* will be used for the rest of the experiment (108 infected trees total, 27 buffer-inoculated). Trees will be allowed to go dormant in screen cages outside.

In December 2005, plants will be divided equally between treatments. One-third will remain outside in the field, 1/3 will be kept at 7°C (45°F), and 1/3 at 1.7°C (35°F). *Xf* dies at these temperatures in grapevines (Almeida and Purcell 2003c, Feil and Purcell 2001). Trees will be removed from each cold treatment at intervals of 1, 2 and 4 months, and allowed to break bud in the greenhouse. These intervals are reflective of dormancy periods used in previous studies with almonds and grapevines (1 mo.; Almeida and Purcell 2003c, Feil and Purcell 2001), typical dormancy in the Central Valley (2 mos.; going fully dormant in December and flowering in February) and an extreme treatment for abnormally long dormancy (4 months). Plants will be kept the greenhouse until they develop almond leaf scorch symptoms, and then sampled via culture as previously described.

CONCLUSIONS

As this is the first year of a three-year study, with the over wintering portion of the treatment yet to be applied, it would be premature to draw conclusions from our data at this time. The effect of overwintering conditions on *Xf* infections will be determined by culturing in summer 2006. Inoculations for plants where *Xf* was not recovered will be repeated in May 2006, and isolations will be repeated in August and September 2006 and 2007. These preliminary results were collected at UCD and the IRC in August and September 2005. Further samples remain to be taken from UC Davis and Kearny Agric. Center.

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IMPORTANCE OF GROUND VEGETATION IN THE DISPERSAL AND OVERWINTERING OF *XYLELLA FASTIDIOSA*

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ABSTRACT
Our goal is to determine the ability of alternate host plants, specifically "ground vegetation," in or near almond orchards or vineyards to serve as reservoirs for *Xylella fastidiosa (Xf)*. We surveyed ground vegetation in ALS-infected almond orchards in California's Central Valley. Plant tissue samples were collected throughout a 2 year period and processed for *Xf* presence using restriction enzyme digestion of RST31-RST33 polymerase chain reaction (PCR) products and bacterial culture on selective media. Overall incidence of *Xf* was low in the ground vegetation species, only 63 of 1369 plant samples tested positive. Of the 37 species of common ground vegetation sampled, 11 tested positive for *Xf*, including such common species as Sheperd's purse (*Capsella bursa-pastoris*), filaree (*Erodium* spp.), cheeseweed (*Malva parvifolia*), burclover (*Medicago polymorpha*), annual bluegrass (*Poa annua*) London rocket (*Sisymbrium irio*), chickweed (*Stellaria media*). There was a seasonal component to bacterial presence, with positive samples found only between November and March. Both ground vegetation and almond trees were most commonly infected with the almond strain of *Xf* (6 of 7 surveyed sites). ALS-infected almond samples had a *Xf* concentration within reported ranges, however, we were unable to accurately measure *Xf* titer in sampled ground vegetation for comparison.

INTRODUCTION
The epidemiological factors of Pierce's disease (PD) and almond leaf scorch (ALS), which lead to economic damage, requires more than the presence of susceptible crop cultivars, insect vectors, and plant species suitable for vector feeding and/or breeding. After the pathogen, *Xylella fastidiosa* (*Xf*) is inoculated into a host plant, bacterial multiplication, systemic movement, and expression of disease symptoms depends on many factors including temperature, date of infection, and the *Xf* strain and concentration. We hypothesize that a reservoir population of *Xf* can reside in and around grape or almond orchards without the outward expression of plant disease. *Xf* reservoirs in adjacent vegetation may increase the window of vulnerability for nearby susceptible crops to become infected by providing enough inoculum for vectors at critical periods. For this reason, removal of blue green sharpshooter breeding hosts was an effective method for controlling the spread of PD in coastal wine grape regions. As yet, there have not been similar studies of vegetation management for controlling the spread of ALS, which has been increasing in prevalence and severity in California's interior valleys. By identifying the seasonal presence and incidence of *Xf* in common ground vegetation in or near almond orchards, weed control efforts can appended to also reduce reservoir *Xf* host species and reduce the level of bacterial inoculum.

We report here are sample collections of annual plant species in almond orchards, where ALS incidence had been recorded for more than 2 years (all sites reporting PD and/or GWSS were heavily treated with insecticides in 2005).

OBJECTIVE
1. Determine the presence of *Xf* in alternate host plants that are commonly visited by glassy-winged and native sharpshooters in selected ecosystems in the San Joaquin Valley; with samples representing different seasons and annual or perennial hosts.

RESULTS
Ground vegetation survey
Surveyed almond orchards were located in California's north Central Valley (Butte Co., Glenn Co.), the middle of the Central Valley (Stanislaus Co.) and the south Central Valley (Kern C.). Every 2 to 6 weeks, depending on the seasonal availability of ground vegetation, a visual survey and collection of the four most abundant weed species was conducted. A total of 58 collections were made. There were 37 species of ground vegetation commonly found (Table 1), with most material collected in winter and spring when ground vegetation was common.

Bacterial detection and strain identification
Each sample (orchard site, sample date, plant species, n = 1369) was processed separately for the presence of *Xf*, using immunocapture DNA separation and PCR amplification procedures developed by B.C. Kirkpatrick (UC Davis, pers. comm.). After gel electrophoresis, a preliminary strain difference analysis was carried out according to Minsavage et al. (1994).

Sixty three of 1369 samples from the six orchards were positive for *Xf* (4.6%). *Xf* was recovered from 11 of the 37 ground vegetation plant species, including 5 species from which it had not previously been recovered in the field (Table 1). There was a strong seasonal component to bacterial presence in ground vegetation, with no *Xf* positive samples found between April and mid-October during the two years of the study (Figure 1). Results from both PCR and culture on selective media showed that almond trees in 6 of 7 experimental orchards were infected with the almond strain of *Xf*. At one site (Stanislaus Co.), a grape strain of *Xf* was isolated from all weeds and almond trees sampled. At each site, tissue samples from both almond trees and surrounding weeds was either the grape or almond strain of *Xf*, but never both.

Bacterial titer and incidence
Attempts were made to culture *Xf* from symptomatic almond trees, as well as fresh samples of alternate host plants, using procedures described by Hill and Purcell (1997), in order to determine both the strain and concentration of bacteria in almond and ground vegetation samples.

Petioles from ALS infected almond trees containing the grape strain of *Xf* had an average concentration of 2.15×10^7 CFU/g, which is significantly greater than the concentrations at other sites sampled (1.84×10^6 - 1.19×10^7 CFU/g) ($P = 0.014$). Previous studies also showed average *Xf* titer in ALS-symptomatic almond leaves (Almeida and Purcell 2003) is lower than the average *Xf* titer in PD-symptomatic grapes (Hill and Purcell 1997). All ground vegetation samples were contaminated with other bacteria species and *Xf* presence could be determined. Previous researchers have also encountered difficulty in culturing *Xf* from field samples (Wistrom and Purcell 2005).

Table 1. Presence of *Xylella fastidiosa* in ground vegetation in ALS-infected almond orchards (using immunocapture DNA extraction and PCR) in this study are compared against previous field surveys near PD-infected vineyards, except for references marked * which refer to greenhouse studies [1].

Scientific Name (Common Name)	This study	Other studies	Reference
Capsella bursa-pastoris (Shepherd's purse), *Senecio vulgaris* (common groundsel), *Sisymbrium irio (*London rocket), *Stellaria media* (Chickweed), *Urtica urens* (burning nettle), *Veronica persica* (Speedwell),	+	None	
Chamaesyce maculate, (spotted spurge), *Chenopodium album*, (lambsquarter), (*Conyza bonariensis*, (fleabane), *Coronopus didymus*, (lesser swine cress), *Festuca spp.*, (fescue grass), *Ranunculus spp.*, (buttercup), *Salsola tragus*, (Russian thistle), *Typha spp.* (cat tail)	-	None	
Erodium spp. (filaree)	+	+	2* , 4*
Medicago polymorpha (burclover), *Poa annua* (annual bluegrass)	+	+	2
Erodium spp. (filaree), *Sonchus spp.* (sowthistle), *Malva parvifolia* (cheeseweed),	+	-	1
Avena fatua (wild oat), *Cyperus esculentus* (yellow nutsedge), *Escallonia montevidensis* (escallonia), *Hordeum murinium* (hare barley), *Rumex crispus* (curly dock)	-	+	2*
Brassicaceae spp. (mustards), *Helianthus spp.* (sunflower)	-	+	1
Claytonia perfoliata (miner's lettuce)	-	+	3
Amaranthus spp. (pigweed), *Conyza canadiensus* (horseweed), *Echinochloa crus-galli* (barnyard grass), *Lactuca serriola* (prickly lettuce), *Portulaca oleracea* (common purselane), *Sonchus oleraceus* (annual sowthistle), *Xanthium strumarium* (cocklebur)	-	+	4*
Amaranthus spp. (pigweed), *Amsinckia spp.* (fiddleneck), *Anagallis arvensis* (scarlet pimpernel)	-	-	1
Lactuca serriola (prickly lettuce), *Sorghum halepense* (Johnson grass)	-	-	3
Portulaca oleracea Common purselane	-	-	2*

[a] References cited are 1 = Costa et al. 2004, 2 = Freitag 1951, 3 = Raju et al. 1983, 4 = Wistrom and Purcell 2005

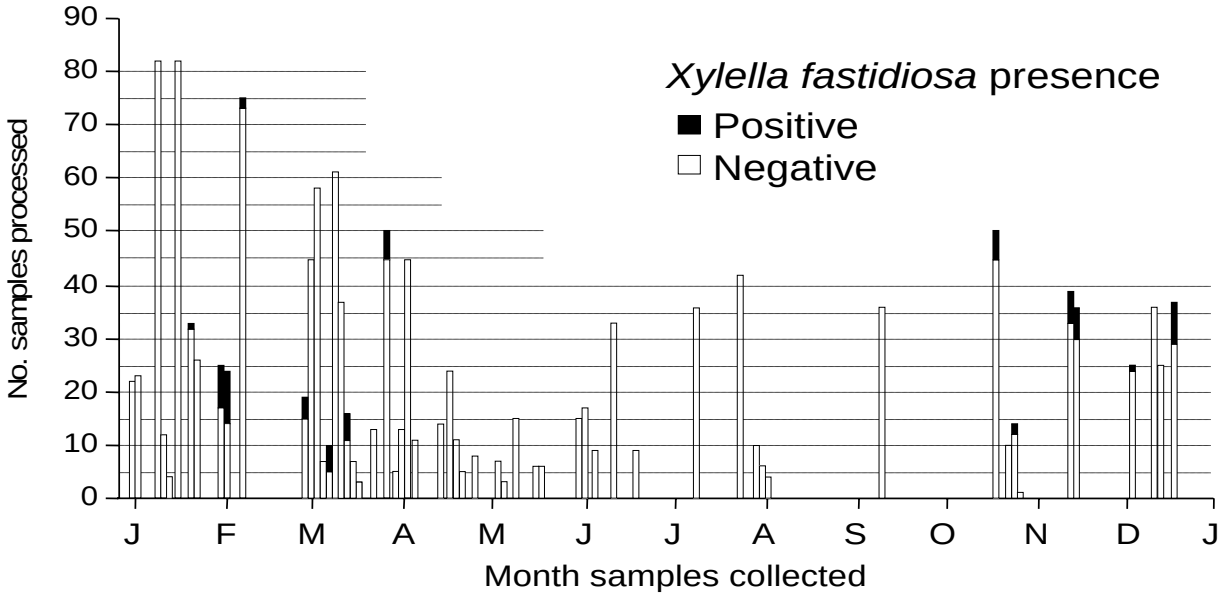

Figure 1. Survey of vegetation in almond orchards for *Xf*. Data show combined results from six almond orchards in Butte, Glenn, Stanislaus, and Kern Counties from June 2003 to April 2005.

CONCLUSIONS

All previous field surveys for *Xf* in alternate host plants have focused on PD management. With the recent increase of ALS in California, there was an even greater need to survey plants in almond orchards for *Xf*. This will be of prime importance as GWSS moves northward into areas dominated by nut and vineyard crops. We showed the presence of *Xf* were present in 29.7% of the ground vegetation species sampled. Numerous studies have documented the survival of *Xf* in different plant species; however, fewer have included field surveys (but see Raju et al. 1983, Hopkins and Alderz 1988, Costa et al. 2004), or the season-long incidence of *Xf* in non-symptomatic ground vegetation.

Of the *Xf* positive plant species in our survey, 9 of the 11 were present in the orchards on most of the sampling dates and thus comprised the largest sample sizes of all ground vegetation species. There was a positive and significant relationship between the number of samples taken per plant species and the percentage of samples positive for *Xf* ($y = 0.0553x - 0.2074$, $r^2 = 0.8935$). Some plant species in the sampled orchards were common hosts of *Xf* in other surveys, but were negative in our 2 year survey (Table 1).

We found the almond strain of *Xf* was most common in the surveyed ALS-infected orchards. Recent studies on the biology of different strains of *Xf* have shown varying abilities to infect different hosts (e.g., Almeida and Purcell 2003). A recent study near Fresno, California, showed that characteristics of different varietals of almonds as well as strain type result in differing severity of ALS (Groves et al. 2005). A parallel study found both the almond and grape genotypes of *Xf* in the same plant, pointing out the presence of a less virulent strain does not preclude the existence of a more virulent strain (Chen et al. 2005). We found significantly higher *Xf* titers in almond petioles containing the grape strain, as compared to petioles with almond strain *Xf* ($P < 0.014$), as has been reported previously (Hill and Purcell 1997, Almeida and Purcell 2003).

Perhaps most important for the relationship between ALS and PD epidemiology and resident ground vegetation is that we detected *Xf* in weeds only between October and April. Other field surveys, conducted primarily during the growing season, detected *Xf* during the summer (Costa et al. 2004, Freitag 1951, Wistrom and Purcell 2005). Seasonality and temperature is important for ALS or PD epidemiology as *Xf* survives best in the plants at a moderate temperature and plants inoculated on leaf tissue late in the growing season may not develop chronic disease symptoms. Ground vegetation in the surveyed orchards best harbored *Xf* at a temperature that was most consistent during the winter months, and when these fall/winter ground covers were newly formed and in good condition. A possible reason for the difference between these studies is that, during the late spring and summer months, most ground vegetation in the almond orchard was small and in poor condition due, in part, to almond management practices of preparing the orchard floor for harvest operations. Therefore, cultural practices may also impact *Xf* levels in alternative host plants.

These results suggest further investigation of the seasonal presence and concentration of *Xf* in ground covers with the seasonal presence and abundance of potential insect vectors. Unlike in vineyards where a clear edge effect has been found with PD incidence, most previous work has not revealed any clear spatial patterns with ALS. As ground vegetation can harbor *Xf* on the almond floor, our results suggest that a year-round vegetation management may assist in PD or ALS

management. Also, the feeding behavior and plant preference of insects could be a more important factor in controlling the spread of PD and ALS.

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DESIGN OF CHIMERIC ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF *XYLELLA*

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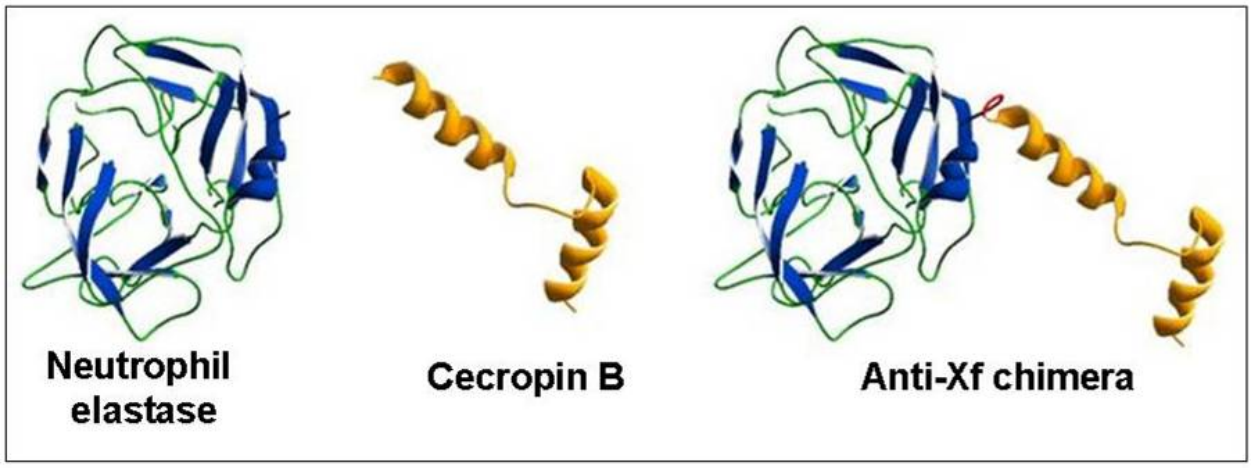
ABSTRACT
Xylella fastidiosa (*Xf*) is a gram-negative xylem-limited bacterium and causative agent of Pierce's disease (PD) in California grapevines. During very early stages of *Xf* infection, specific carbohydrates/lipids/proteins on the outer membrane of *Xf* interact with plant cells and are important for virulence (1). Design of a protein inhibitor that interrupts this step of the plant-*Xf* interaction will be useful in anti-microbial therapy and controlling PD. In this UC/LANL project, we propose a novel protein-based therapy that circumvents the shortcomings of an antibiotic. We have designed a chimeric anti-microbial protein with two functional domains. One domain (called the surface recognition domain or SRD) will specifically target the bacterium outer-membrane whereas the other will lyse the membrane and kill *Xf*. In this chimera, human neutrophil Elastase (5-10) is the SRD that recognizes MopB, the major outer membrane protein of *Xf* (11). The second domain is Cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have combined Elastase and CecropinB using a flexible linker such that both components can simultaneously bind to their respective targets. This chimeric gene was synthesized and cloned into different vectors for insect and plant transformation. Five transformed insect cell lines are being evaluated and production and processing of the protein is being optimized in in liter size preps. Plant transformation experiments have been completed and we have obtained plants of *Nicotiana tabacum* var *benthamiana* and plants of *Vitis vinifera* 'Thompson Seedless' transformed with this gene that are currently being analyzed for gene expression and protein production. The proteins obtained from the transgenic insect and plant cell lines will be used to test for antimicrobial activity against *Xf*.



INTRODUCTION
Globally, one-fifth of potential crop yields is lost due to plant diseases primarily of bacterial origin. *Xylella fastidiosa* (*Xf)* is a devastating bacterial pathogen that causes PD in grapevines, citrus variegated chlorosis (CVC) in citrus, and leaf scorch disease in numerous other agriculturally significant plants including almonds in California (http://danr.ucop.edu/news/speeches). Since the glassy-winged sharpshooter (an insect vector) efficiently transmits PD, a great deal of effort has been focused on using insecticides to localize and eliminate the spread of this disease. However, the

availability of the whole genome sequences of PD and CVC strains of *Xf* offer new avenues to directly target and inactivate the pathogen. In this project, we propose a structure-based approach to develop chimeric anti-microbial proteins for rapid destruction of *Xf*. The strategy is based upon the fundamental principle of innate immunity that plants recognize and clear pathogens in rapid manner (1-2). Pathogen clearance by innate immunity occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction by anti-microbial processes. Different sets of plant factors are involved in different steps of innate immunity. Our strategy of combining a pathogen recognition element and a pathogen killing element in the chimeric molecule is a novel concept and has several short and long term impacts.

OBJECTIVES

Objective 1:

a) Utilize literature data and computer modeling to identify an SRD that specifically targets MopB (Elastase)

b) Utilize literature data and computer modeling to identify a useful Cecropin (i.e., Cecropin B)

c) *In vitro* testing of anti-*Xylella* activity of the MopB-specific SRD (Elastase) and Xyllela-specific Cecropin B and demonstration of synergistic killing effect due to the combined use of Elastase and Cecropin B.

Objective 2:

a) Design and construction of synthetic gene encoding Elastase-Linker-Cecropin B Chimeric protein.

b) Expression Elastase-Linker-Cecropin B in insect and plant cells and testing activity *in vitro*.

Objective 3:

a) Expression in transgenic plants

b) Testing for anti-*Xylella* activity *in planta* and testing for graft transmissibility.

RESULTS

Following our successful accomplishment of Objectives 1a, b & c in the first year of our project, where functional activity of Elastase (SRD for MopB) and Cecropin B (defensin) components were tested individually, we designed a chimeric protein of Cecropin B and HNE (Objective 2a). The covalent attachment of Cecropin B to HNE is proposed to increase the stability of the peptide by lowering the conformational entropy of its unfolded state and to increase the overall affinity for the bacterial surface by minimizing the degrees of motion at the binding site, thereby increasing binding between the ligands and the surface.

The HNE-Cecropin B chimera gene was synthesized and cloned into pBacPAK8 baculovirus vector. The chimeric gene inserted into pBacPAK8 was co-transfected with BacPAK6 viral DNA into Sf21 cells. Recombinant viruses formed by homologous recombination were amplified, and the protein expression was optimized in High Five cells (Invitrogen, Carlsbad, CA), derived from *Trichoplusia ni* egg cell homogenates. High Five cells have been shown to be capable of expressing significantly higher levels of secreted recombinant proteins compared to Sf9 and Sf21 insect cells. Optimal conditions for the expression have been worked out in HighFive cells; suspension cells in logarithmic growth are infected with recombinant *Xf* chimera baculovirus, with a multiplicity of infection of 10, and grown for 72 hours. About 25-50% of the expressed chimeric protein is secreted into the supernatant and is detected on a Western Blot as a single band. The supernatant is collected, concentrated and dialyzed. Concentrated supernatant is then run on a weak cation exchange column, chimeric protein containing fractions are pooled and dialyzed, and the dialyzed fractions are run on an elastin affinity column. All chromatography steps are carried out by gravity flow. Chimeric protein containing fractions are pooled and dialyzed and tested for elastase activity. By these methods, we are able to purify ~250 □g active protein from 50mL supernatant. These conditions are being scaled up to produce the amounts required for testing against *Xylella fastidiosa* (currently purifying liter size preps).

We have also cloned the chimera into a plant vector (Figure 1) that was electroporated into disarmed *Agrobacterium tumefaciens* strain EHA 105 creating a functional plant transformation system that has been used to transform pre-embryogenic callus of *Vitis vinifera* 'Thompson Seedless' and the rootstock 'Freedom'.

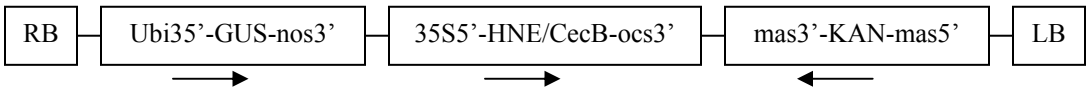


Figure 1. Schematic representation of binary plasmid pDU04.6105

We have obtained more than 40 seedlings of 'Thompson Seedless' from independent lines and expect that, based in our experience with grape transformation, the majority of them will develop into normal plants. Those plants will be micropropagated and acclimated in the greenhouse and analyzed for gene expression, PD tolerance and graft transmissibility.

In addition, the same experiments have been performed using a second construct in which the coding sequence of the signal peptide of HNE was replaced with that of the pear polygalacturonase inhibiting protein (pPGIP). The aminoacid sequence of this chimeric gene product is shown in Figure 2. Our hypothesis is that the pPGIP signal peptide will direct/improve the secretion of the chimeric protein and, as a consequence, increase its concentration in the xylem. This hypothesis in based in previous results that have shown that the product of the pPGIP encoding gene, heterologously expressed in transgenic

grapevines, is present in xylem exudates and moves through the graft union (14). Leaf discs of *Nicotiana tabaccum* 'benthamiana' and 'RT1' have also been transformed with HNE-Cecropin and pPGIP-HNE-Cecropin B genes. The plants obtained are currently being analyzed for gene expression.

*MELKFSTFLSLTLLFSSVLNPALS***IVGGRRARPHAWPFMVSLQLRGGHFCGATLIAPNFVMSAAHCVANVNVRAV RVVLGAHNLSRREPTRQVFAVQRIFEDGYDPVNLLNDIVILQLNGSATINANVQVAQLPAQGRRLGNGVQCL AMGWGLLGRNRGIASVLQELNVTVVTSLCRRSNVCTLVRGRQAGVCFGDSGSPLVCNGLIHGIASFVRGGCA SGLYPDAFAPVAQFVNWIDSIIQ**GSTA*KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL***

Figure 2. pPGIP-HNE-Cecropin B chimeric amino acid sequence. The signal peptide of HNE (MTLGRRLACLFLACVLPALLLGGTALASE*)* has been replaced with the predicted signal peptide of pPGIP (italics) which has been fused to the N-terminal of the mature HNE (bold). HNE is attached to Cecropin B (bold italics) by the GSTA linker, which is underlined.

CONCLUSIONS

The main objective of this project is to develop a potent therapy against *Xf* by utilizing the principles of innate immunity by which plants recognize pathogens using their surface characteristics and then rapidly clear them by cell lysis. We have developed a chimeric anti-microbial protein containing two functional domains. One domain (called the surface recognition domain or SRD) will specifically target the *Xyllela* outer-membrane whereas the other will lyse the membrane and kill *Xyllela*. In this chimera, elastase is the SRD that recognizes mopB, the major outer membrane protein of *Xf*. The second domain is cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have successfully tested each of these components individually and demonstrated that they each (elastase and cecropin B) display activity against *Xf*, which is synergistic when both proteins are combined. We have tested the protease activity of elastase against the purified mopB and intact *Xf* cells to demonstrate that the *Xylella* protein is degraded and therefore, a target for elastase. We have successfully combined the elastase and cecropinB using a flexible linker such that both components can simultaneously bind to their respective targets. This chimeric gene has been synthesized, cloned into a pBacPAK8 baculovirus vector, and packaged into recombinant baculovirus in Sf21 insect cells. Optimization of chimeric protein production is ongoing. We have also transformed pre-embryogenic callus of *V.vinifera* L. 'Chardonnay' and 'Thompson Seedless' and the rootstock 'Freedom'. Transgenic callus will be cultured in bioreactors designed to optimize protein production by secretion into the medium. We plan to use this system as well as the insect bioreactors to validate the anti-*Xylella* properties of the chimeric protein. Transgenic plants will be obtained from transgenic callus cultured in germination medium. After acclimation in the greenhouse, they will be inoculated with *Xf* and tested for PD tolerance/resistance.

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EVALUATION OF GRAPEVINE ENDOPHYTIC BACTERIA FOR CONTROL OF PIERCE'S DISEASE

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ABSTRACT
We continued to screen our endophyte library for *in vitro* antagonism of *Xylella fastidiosa* (*Xf*) growth. Approximately 16 isolates exhibited antagonism out of the 150 strains that were screened. To date, we have screened approximately 650 isolates and identified 66 that showed some level of *Xf*-antagonism. We are continuing to screen the rest of the library and will conduct grapevine movement assays on all antagonists in the coming year.

Greenhouse testing of six grapevine endophytes that began in 2003 showed that three isolates provided statistically significant reduction in Pierce's disease (PD) symptom severity. Five months after these vines were removed from the greenhouse and planted in the field, all but one of the non-protected, *Xf*-inoculated, positive control vines were dead or had PD symptoms. No symptoms were observed in any of the ten vines that were inoculated with a *Cellulomonas* endophyte nor eight of ten vines inoculated with a *Bacillus* spp. These results indicate that these two endophytes have the ability to suppress populations of *Xf* within grapevines, because these vines initially tested positive for *Xf* four months following inoculation nor after transplanting these vines in the field.

A large greenhouse biocontrol experiment involving four of the Pseudomonads, or combinations of the Pseudomonads with *Pseudomonas viridiflava* to act as a movement facilitator, were established in August 2004. Unfortunately it appears that there was a low efficiency of inoculation using blue-green sharpshooters (BGSS) as well as mechanical inoculation because PD symptoms were not evident on the positive control vines four months following inoculation. The vines will be rated again for symptoms and planted in the field, which may help to induce PD symptoms.

Additional endophytes were isolated from "escape" vines; i.e. vines without apparent symptoms in vineyards with large PD losses on two occasions in Fall 2004. Representative colonies were grown in liquid media and stored at -80°C. During 2005 these isolates, as well as others from the original endophyte library will be screened for *Xf*-antagonism *in vitro*.

INTRODUCTION
Xf, the bacterium that causes PD, colonizes only xylem vessels, an ecologically distinct niche which supports the growth of comparatively few microbes. However, previous research conducted in Nova Scotia and in our lab has shown that a number of bacterial species can be isolated from grapevine xylem sap. While some of these bacteria are most likely wound inhabitants that cannot systemically colonize xylem elements, other species have been shown to move over 30cm, a distance that likely would involve the active degradation of xylem pit membranes that separate individual xylem elements. Previous research showed that some of the systemic colonizers were antagonistic to *Xf* in *in vitro* growth inhibition assays (see previous progress reports). Greenhouse grown grapevines that were inoculated with some of these strains did not prevent initial multiplication of mechanically inoculated *Xf*, however some of these strains prevented the subsequent development of PD (see below). If additional testing substantiates the protective properties of these bacterial endophytes against PD, these strains may provide a novel and environmental benign approach for minimizing losses to PD.

OBJECTIVES
1. Finish evaluating our existing library of grape endophytic bacteria to identify antagonists of *Xf in vitro*.
2. Evaluate the biocontrol abilities of endophytes against *Xf* including:
   i) prevention of infection
   ii) suppression of PD symptom development
   iii) long term health and survival of infected vines in field experiments.
3. Isolate additional endophytes from asymptomatic vines in infected vineyards (escape vines) and characterize these isolates for antagonistic traits.

RESULTS AND CONCLUSIONS
Continue in vitro screening the library of grape endophytic bacteria to identify antagonists of *Xylella fastidiosa* growth.
Details regarding the methods we used for the *in vitro* antagonism assay have been presented in previous progress reports. In brief, 100ul of a 10^8 CFU culture of the Fetzer or Temecula strain of *Xf* are spread over a plate of solid PD3 medium. A small amount of each endophyte isolate is removed from the -80°C glycerol stock and streaked out on the medium in which it was originally isolated to obtain single colonies. The *Xf* plates are incubated for approximately four days and then a small amount

of the endophyte is applied to the center of the *Xf* plate. The plate is then incubated for seven days until *Xf* colonies are clearly visible. If the endophyte has the ability to inhibit the growth of *Xf,* the size of the inhibition zone is measured and recorded.

During this period we screened approximately 150 more endophytes, bringing the total number of screened isolates to 650, which is approximately 2/3 of the total number of isolates that Dr. Darjean collected. Approximately 16 of the 150 isolates showed some degree of antagonism towards *Xf* growth *in vitro*. This brings the total number of endophytes that exhibited some degree of *in vitro* antagonism towards *Xf* to 66, or approximately 1/10 of the isolates that were screened. If this trend continues we would expect to identify approximately 100 endophytes that exhibit some degree of antagonism towards *Xf* from the entire library.

Table 1. Grapevine endophytes screened during 2004 that showed some degree of antagonism towards *Xf*.

Endophyte	RFLP Group	Zone of clearing
75	37	25 mm
110	15	25 mm
122	15	6 mm
127	15	10 mm
128	15	10 mm
138	15	15 mm
145	16	5 mm
174	Group 28	slower/complt
176	28	18 mm
178	19	6 mm
184	Group 80	3 mm
197	Group 7	20 mm
200	Group 7	5 mm
220	7	slower growth
221	7	5mm
310	33	10mm

Assess the ability of antagonists to colonize and move systemically in grape xylem.
Prior to her departure in October, 2004, Dr. Whisler screened 16 of the *Pseudomonas* endophytes for their ability to move in Chardonnay grapevines growing in the greenhouse. The vines were trained as a single cane and the cane was grown to approximately 1m. The endophytes were suspended in phosphate buffer to a density of approximately 10^7 CFU/ml and approximately 20ul of the suspension was pinprick inoculated into the stem using the same methods that we use to mechanically inoculate vines with *Xf*. Two grapevines were inoculated with each strain. After six weeks of growth in the greenhouse, 1cm stem sections were removed at 10cm and 30cm above the point of inoculation. The second petiole above the point of inoculation was also removed to assess whether the endophyte had the ability to cross the xylem pit membrane and enter into the petiole. The stem sections and petiole were surface sterilized in 10% bleach and 80% ethanol for 1 min each and then rinsed three times in sterile di-water. The stem sections were placed in sterile grinding bags with 2 mls of sterile phosphate buffer and the tissue was ground using a ball bearing grinder. One hundred ul of the homogenate was plated on the medium on which the endophyte was originally isolated. Colonies with morphologies that were similar to the inoculated endophyte were counted and one or two representative colonies were chosen at random for strain identification. As described in previous progress reports, the 16S rDNA from these colonies was PCR-amplified and sequenced to verify the identity of the putative endophyte. Table 2 summarizes the results of the movement assays for these 16 isolates.

Table 2. Movement and other characteristics of 16 Pseudomonad grapevine endophytes.

Pseudomonas subgroup	# of isolates	Vine Health[1]	Antagonism[2]	Movement[3]
1	5	Healthy	Complete[4]	10 cm
2	5	Healthy	Complete[4]	10 cm
3	3	Escape/Healthy	20-25 mm	Petiole/ 30 cm
4	1	Healthy	Complete[4]	10 cm
5	2	Healthy	5 mm	N/D[5]

[1]Condition of vine of origin at time of endophyte isolation.
[2]Antagonism of *Xf* growth is the zone of inhibition or the distance from the endophyte to the visible growth of *Xf*.
[3]Re-colonization and movement in grape xylem was assessed at 10cm and 30cm from the point of inoculation, and from the second petiole above the point of inoculation.
[4]Complete: no growth of *Xf* visible.
[5]N/D: not yet determined.

All of the Pseudomonads moved at least 10cm above the point of inoculation, however work done in the Labavitch laboratory has shown that a small proportion of xylem elements are greater than 10cm in length thus these positive isolations may reflect inoculation into some of the longer elements. Isolates from subgroup 3, which were phylogenically most similar to *Pseudomonas viridiflava*, moved the greatest distance, at least 30cm from the point of inoculation. These isolates were also the only ones recovered from the petiole, suggesting they had the ability to degrade pit membranes that presumably occur between xylem vessels in the stem and the petiole. These isolates were plated on a sodium polypectate medium that can detect the production of polygalacturonase (PG), an enzyme that degrades pectin-like polymers. Clearing zones around the colonies proved that these isolates, like true *Pseudomonas viridiflava* strains produce PG. Ms. Caroline Roper, a student working jointly in the Labavitch and Kirkpatrick labs, has shown that the production of PG is absolutely necessary for the movement of *Xf* in grapevines (2004 PD/GWSS Conference). It would appear that *Pseudomonas viridiflava* would be an excellent candidate as a potential *Xf*-antagonist or it could act as a "movement facilitator" which, when co-inoculated with a stronger *Xf* antagonist, could facilitate the movement of the stronger antagonist by degrading xylem pit membranes.

Continued evaluations of biocontrol experiment initiated by Dr. Darjean-Jones in 2003
A previous PD progress report presented many details about a biocontrol project that was initiated by Dr. Darjean-Jones in 2003. The following provides an update of the field evaluation of these plants that was done in October 2004 and 2005.

Six bacterial grapevine endophytes that exhibited antagonism to *Xf in vitro* and which moved 15cm from the point of inoculation into grapevines growing in the greenhouse were evaluated as potential biocontrol agents for PD. Each strain was inoculated into 10 Cabernet Sauvignon vines in April, 2003 and allowed to colonize the vines for six weeks in the greenhouse. With the assistance of Purcell's group at UC Berkeley, the vines were then exposed to *Xf*-infectious BGSS. The vines were returned to UC Davis and kept in a greenhouse. Four months later, in September 2003, they were tested for *Xf* by IC-PCR and their symptoms were rated on a severity scale of 0 (healthy) to 4(dead). These results are shown below:

Table 3. Greenhouse evaluation of endophyte vines four months following *Xf* inoculation (10 reps each endophyte).

Endophyte Inoculated	*Xf* PCR (+)	Average Disease Severity (0-4)[z]
Bacillus megaterium	9/10	2.0
Streptomyces spp	7/10	2.3
Bacillus spp –147	9/10	1.5*
Bacillus spp –161	9/10	1.4*
Cellulomonas	9/10	1.5*
Agrobacterium $F_2 5$	10/10	2.2
Control, no endophyte	9/10	2.1

z. disease severity calculated with PCR (+) only. 0=healthy; 4=dead.
* statistically significant difference at p=0.05.

PCR analysis showed that: 1) there was a high rate of successful transmission using the BGSS and 2) none of the endophytes provided protection against initial infection by *Xf*. However, three of the endophytes provided a statistically significant reduction in the severity of PD symptoms after four months of growth in the greenhouse. These vines were kept in the greenhouse over the winter, during which time some of the vines died from PD. In spring 2004, all of the remaining vines were planted in the field at UC Davis. The vines were fertilized and watered with a drip system. In October 2004 and 2005 the vines were rated for PD symptoms. Table 4 presents those results.

Table 4. Disease evaluation of endophyte-inoculated vines planted in the field approximately 1 ½ and 2 ½ years following inoculation with *Xf*.

Endophyte Inoculated	Healthy/Vine Vigor[z]		PD Symptomatic		Dead
	October 04	October 05	October 04	October 05	
Bacillus megaterium	0/NA	1/3	1	0	9
Streptomyces spp.	5/2.6	6/2.5	1	0	4
Bacillus spp –147	6/2.5	6/2.8			4
Bacillus spp –161	8/2.8	8/2.4	1	0	1
Cellulomonas	10/2.8	10/2.8			0
Agrobacterium F_25	5/2.5	5/3			5
Control, no endophyte	1/1.8		2	2	7

z. Vigor rated on a scale of 3= comparable to other non-endophyte inoculated vines; 1= poor growth

The *Cellulomonas* and *Bacillus*-161 strains provided good suppression of PD symptoms in the field. Petioles from these vines also tested negatively for *Xf* by PCR while symptomatic leaves from some of the other vines tested positively for *Xf*. This would suggest that these strains greatly suppressed the growth of *Xf* from the time when they tested positive four months following inoculation to the time they were tested one and two years later. Xylem sap from a few of the *Cellulomonas*- and *Bacillus*-inoculated vines was plated on endophyte media and some colonies that morphologically resembled these strains were seen. However, the identity of these colonies was not proven by analysis of their 16S rDNA. Additional xylem sap has been extracted from these vines and identity of isolated bacteria is now being done by a new graduate student, Margot Wilhelm. We will re-inoculate these endophyte strains into young and five year old Cabernet Savignon and Chardonnay, which is more susceptible to PD, in 2006. Budwood will be collected from the *Cellulomonas* and *Bacillus*-161 vines during the winter and rooted in spring 2006. Xylem sap will be examined for the presence of the endophytes in some of the rooted vines while others will be mechanically inoculated with *Xf* to determine if propagated vines possess any resistance to PD.

2004 biocontrol experiment initiated by Dr. Whistler

A large biocontrol experiment was initiated by Dr. Whistler beginning in July 2004. This experiment focused on the Pseudomonad group 7 that exhibited good *in vitro* inhibition. There were a total of 24 treatments with 10 Chardonnay vines per treatment. Four strains, 197 (*Pseudomonas viridiflava*, a strong grapevine colonizer), 205, 329 and 403 (strains that strongly inhibited *Xf in vitro*) were individually inoculated in 10 vines/trmt using the pinprick inoculation procedure routinely used to inoculate *Xf*. In addition, a movement facilitator treatment using strain 197 in combination with 205 or 329 or 403 was also inoculated into 10 vines/trmt. To assess the potential impact of the endophyte on grapevine growth, the strains were also individually inoculated into 10 vines/trmt. Ten vines of each endophyte individual or combination treatment were mechanically inoculated with *Xf* and ten vines of each treatment were insect inoculated using putatively infectious BGSS in cooperation with Sandy Purcell's lab. Ten vines not inoculated with anything served as controls to monitor greenhouse environmental conditions. Ten vines each were inoculated with buffer alone, or buffer then *Xf* inoculated mechanically or with BGSS. In total this experiment had 240 potted vines.

Unfortunately, subsequent infectivity tests by the Purcell lab found that the batch of BGSS that was used to inoculate the vines had poor transmission rates to test plants kept at Berkeley. Because of the long latent period, typically 12 to 14 weeks, for PD symptoms to show, we did not know these results until it was too late to acquire more BGSS for another inoculation attempt. In addition, the mechanically inoculated, positive control vines still appeared healthy in December 2004 and April 2005, three and seven months after *Xf* inoculation. In May 2005, the vines were transplanted into the field in the hope that PD symptoms would develop in the fall. Unfortunately none of the non-protected, positive control vines developed symptoms, which indicates that, for reasons unknown, the *Xf* inoculation was unsuccessful. In addition, because the vines were inadvertently sprayed with *Bacillus thurengensis* (BT) to control a caterpillar infestation and BT has very resilient endospores, attempts to at least measure how effectively the endophytes colonized the control vines were ruined because

surface sterilization failed to kill the BT spores and isolation plates were completely contaminated with BT. This was obviously a great disappointment for all involved. Because the Pseudomonads looked so promising in the initial screening process we will repeat this screen in 2006.

Isolate additional endophytes from asymptomatic vines in vineyards with a high incidence of PD

Two isolations from 10 "escape vines" were made in late August and early October 2004 in order to verify that the vines were truly asymptomatic. Xylem sap was expressed from these vines using the pressure chamber as previous described. Aliquots of the sap were plated on the same media that we have used throughout this study. Representative colonies were individually grown in liquid medium, the culture was adjusted to 15% glycerol and frozen and -80°C. In 2006, we will screen these isolates for anti-*Xf in vitro* activity in the manner previously described.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

IDENTIFICATION OF MECHANISMS MEDIATING COLD THERAPY OF *XYLELLA FASTIDIOSA*- INFECTED GRAPEVINES

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Reporting Period: The results reported here are from work conducted October 2004 to September 2005.

ABSTRACT
During October/November 2004, 11 control and 11 *Xylella fastidiosa* (*Xf*) infected Pinot Noir and Cabernet Sauvignon grapevines grown in five gallon pots were transported to four locations in Northern California with different winter severities. Another set of similar healthy and *Xf*-infected vines were placed in four different cold rooms with varying temperatures. These vines were rated for mortality and remission of Pierce's disease (PD) symptoms in fall 2005. A high level of mortality occurred at the coldest location (Fall River, Shasta County), moderate mortality occurred at UC Blodgett Research Station, while little mortality occurred at the UC Davis and Hopland sites. Disease ratings were lower in vines from the three cold temperature sites compared to vines grown at UC Davis, however the large discrepancy in the number of vines surviving at each location prevented meaningful comparisons between the field sites. Unexpectedly, mortality of vines in the warmest and coldest chamber regimes was greater than the two intermediate temperatures. Disease severity was also greatest in the warmest temperature which may have contributed to the observed high mortality when the vines were subsequently planted in the field. All three cold chamber regimes had lower disease ratings than the warmest temperature.

Comparisons of xylem sap pH and osmolarity in Cabernet vines growing in a vineyard in Placer County and UC Davis were not consistent with results obtained in 2004. Differences in the date of collection may have influenced these results. Effects of buffer and xylem sap on the survival of *Xf* and various cold temperatures were reported in the Proceedings of the 2004 Pierce's Disease Research Symposium. Abscisic acid (ABA) levels are elevated in many cold-treated plants and ABA has been shown to induce the synthesis of certain pathogenesis related (PR) proteins that in some case possess anti-fungal properties. ABA concentrations were lower in xylem sap collected from vines growing in El Dorado County compared to UC Davis, which suggests ABA is probably not directly mediating the cold therapy phenomenon. However, we are proceeding with experiments to determine if exogenous applications of ABA on non-chilled grapevines can elicit PR proteins.

INTRODUCTION
The geographical distribution of PD in North America is strongly associated with the severity of winter temperatures, i.e. PD does not occur in New York, the Pacific Northwest nor at high altitudes in South Carolina, Texas, and California (Hopkins and Purcell, 2002). Sandy Purcell demonstrated that relatively brief exposures to sub-freezing temperatures can eliminate *Xf* in some percentage of cold treated *Vitis vinifera* grapevines; however some of the coldest temperatures he used killed the vines (Purcell 1977, 1980). He also found that a higher percentage of vines that were moderately susceptible to PD such as Cabernet Sauvignon, were cured by cold therapy treatments compared to susceptible varieties such as Pinot Noir. Purcell's group also showed that whole, potted vines exposed to low temperatures had a higher rate of recovery than PD-affected, detached bud sticks exposed to the same cold temperatures (Feil, 2002).

Clearly, some factor(s) expressed in the intact plant, but not in detached bud sticks, helped eliminate *Xf* from the plants. Our objective is to elucidate the physiological/biochemical basis that mediates cold therapy and to identify the physiological/biochemical factor(s) that occur or are expressed in cold treated vines that eliminate *Xf*. If such factor(s) are found, it may be possible to induce their expression under non-freezing temperatures and potentially provide a novel approach for managing PD.

OBJECTIVES
1. Develop an experimental, growth chamber temperature regime that can consistently cure PD affected grapevines without causing unacceptable plant mortality.
2. Analyze chemical changes such as pH, osmolarity, total organic acids, proteins and other constituents that occur in the xylem sap of cold-treated versus non-treated susceptible and less susceptible *V. vinifera* varieties.
3. Assess the viability of cultured *Xf* cells growing in media with varying pH and osmolarity and cells exposed to xylem sap extracted from cold- and non-treated grapevines.
4. Determine the effect of treating PD-affected grapevines with cold plant growth regulators, such as ABA, as a possible therapy for PD.

RESULTS AND CONCLUSIONS

Objective 1

Using the same varieties used by Purcell (1977, 1980) and Feil (2002) in previous cold therapy studies, Pinot Noir (PD-susceptible) and Cabernet Sauvignon (moderately resistant to PD) grapevines grafted onto 101-14 rootstock were inoculated with *Xf* in the spring using a pinprick inoculation procedure (Hill and Purcell, 1995; Purcell and Saunders, 1999). The vines were grown in five gallon pots in a greenhouse using a nutrient-supplemented irrigation regime. Treatment vines were inoculated with the Stagg's Leap strain of *Xf*, whereas control vines were inoculated with water. During late summer and fall, the plants were moved into a screen house in order to acclimatize them to decreasing temperatures. While in the screen house, plants were watered by drip irrigation and supplemental fertilizer application until the first week of October 2004. Twelve weeks after inoculation, the plants were rated for symptom development.

In the spring of 2005, new plants of Pinot Noir and Cabernet Sauvignon grafted on 101-14 rootstock were planted in 5-gallon pots, inoculated by the same procedure used in the spring of 2004 mentioned above. Plants were placed in the same greenhouse, subjected to a similar temperature regime, and were watered using the same nutrient-supplemented regime. Plants were moved to the same screen house as the 2004 plants and will continue to be watered by drip irrigation and receive supplemental fertilizer applications until the first week of October 2005.

During October/November, 2004, 11 inoculated and 11controls of each variety (44 plants total) were transported to three sites that were selected because of their relatively cold winter temperatures, as well as UC Davis, which was the control. Plot sites include: Fall River (Shasta County), UC Hopland Research Station (Mendocino County), and UC Blodgett Forest Research Station (El Dorado County). Potted grapevines were planted in the ground to the top of the pot in order to maintain uniform soil type, prevent roots in the pots from exposure to abnormally cold temperatures, and to prevent the plants from falling over. Plants were irrigated as needed until rain provided adequate moisture for the vines. Vines were allowed to undergo natural dormancy during the fall and experience ambient temperatures during the winter. Temperature, ETo, and other weather data for each plot was monitored using CIMIS weather data (http://wwwcimis.water.ca.gov/cimis/data.jsp). The plants prepared in 2005 will be used to replicate the 2004 study. This data, and previous temperature profiles at these sites, will be used to determine a growth chamber temperature regime that can consistently cure PD affected grapevines without causing unacceptable plant mortality.

Grapevines, using the same varieties and inoculated as described above, but grown in 6" standard pots were exposed to different temperature regimes in cold rooms located at the Department of Pomology, UC Davis during the winter of 2005. Plants prepared in 2004 were subjected to one of four temperature regimes:

Regime 1:	Regime 2:	Regime 3:	Regime 4:
-5°C day; -5°C night	+0°C day; -5°C night	+2.2°C day; -5°C night	+5°C day; -5°C night

There were 40 plants per treatment regime (10 inoculated plants and 10 control plants for both varieties). In regimes where there were differences in day and night temperatures, plants were moved twice daily by carts to simulate daily temperature fluctuations. After three months of treatment, xylem sap was extracted from the plants, and then the plants were moved and planted in the Plant Pathology field at UC Davis. Late in the summer of 2005, the plants were evaluated for symptoms to determine the most effective temperature regime for curing without causing unacceptable plant mortality. The field plant evaluation shows higher disease ratings for the warmer temperature treatments, Davis and Hopland, when compared to the colder treatments, Fall River and Blodgett (Table 1). As we expected, plant mortality was the highest at the colder locations (Table 2). Fall River vines had very high mortality when compared to the other treatments. To try to reduce the mortality in Fall River vines, plants for the 2005-2006 trials will be planted later in the fall to allow the plants to acclimate prior to planting. Cold room treated vines show a similar relationship with the exception of the mortality rate of the +5 day/ -5 night treatment (Tables 3 and 4). This high rate of mortality could be due to rabbits burrowing under the fence and feeding on these plants.

Table 1: Mean PD ratings[z] for PD-infected plants

	Davis	Hopland	Fall River	Blodgett
Pinot Noir	2.17	1.45	1.00	1.33
Cabernet Sauvignon	2.33	2.33	2.00	1.40

[z]Vines were rated for the severity of disease symptoms, 0= healthy to 5= dead.

Table 2: Plant mortality rate

	Davis	Hopland	Fall River	Blodgett
Pinot Noir	0%	2%	91%	41%
Cabernet Sauvignon	0%	2%	55%	36%

Table 3: Mean PD ratings[z] for PD-infected plants

	+5°C Day/ -5°C Night	2.2°C Day/ -5°C Night	0°C Day/ -5°C Night	-5°CDay/ -5°C Night
Pinot Noir	3.00	1.17	1.40	1.00
Cabernet Sauvignon	2.50	1.56	1.29	1.00

z. Vines were rated for the severity of disease symptoms, 0= healthy to 5= dead.

Table 4: Plant mortality rate

	+5°C Day/ -5°C Night	2.2°C Day/ -5°C Night	0°C Day/ -5°C Night	-5°CDay/ -5°C Night
Pinot Noir	45%	27%	32%	59%
Cabernet Sauvignon	14%	9%	0%	14%

Objective 2

Preliminary work from Pinot Noir and Cabernet Sauvignon field materials collected from El Dorado County and Yolo County showed some differences in xylem sap pH and osmolarity (Tables 5 and 6). These results were obtained from Pinot Noir and Cabernet Sauvignon vines growing at Clos des Knoll vineyard in El Dorado County and at the Foundation Plant Services (FPS) vineyard at UC Davis (Yolo County). Both varieties were grown in the same manner at each site; however management practices at the two sites were not identical. It is also important to note that the El Dorado County vines and the Yolo County vines were not the same clones. In 2004, dormant cuttings were collected in late February and xylem sap was extracted using a custom-made pressure bomb. Differences were noted in xylem sap pH, ABA concentration, and osmolarity. These same parameters were examined again in 2005 from grapevines found at the same two locations in late March.

Although only preliminary findings, we found that the pH of xylem sap collected in 2004 in late February was lower, 5.37 for Pinot and 5.23 for Cabernet vines in El Dorado County (colder winter temperatures) than vines growing at FPS (UC Davis), 6.35 and 6.06, respectively. Small differences in osmolarity were also noted in xylem sap from Placerville, 55.2 and 55.5, versus the osmolarity of xylem sap from UC Davis vines, 58.3 and 60.8 respectively. This is different from the xylem sap collected in late March of 2005. The pH of sap from El Dorado County was higher than Yolo County vines. The osmolarity was again similar, but lower at both sites than in 2004. Differences in pH and osmolarity could possibly be due to the difference in timing of collection. The significance and reproducibility of these differences needs to be confirmed with sampling in late February and again in late March of 2006.

In 2004 and 2005, field grown and growth chamber plants prepared as stated in Objective 1, were sampled for potential changes in pH, osmolarity, protein profile and other constituents that occur in xylem sap. Our hypothesis is that changes in xylem sap components in vines that undergo cold treatment may have significant effects on *Xf* viability. Previous research on several plant species has shown that a number of plant genes are expressed in response to freezing temperatures (reviewed by Thomashow, 1998). In some plants, these freeze-induced proteins are structurally related to proteins that plants produce in response to pathogens, i.e. pathogenesis-related proteins (Hon, et al. 1995; Kuwabara, et al, 2002). Thus it maybe possible that cold-stressed grapevines produce proteins that are deleterious to *Xf*.

To investigate this possibility, after three months of treatments, xylem sap was extracted from plants from each location/ treatment, for both growth chamber and field plants, using the pressure bomb. Xylem sap osmolarity and pH were determined for each location/treatment (Tables 7 and 8). We are in the process of concentrating the proteins by acetone precipitation and running the protein precipitate using a 1-dimensional polyacrylamide gel electrophoresis (PAGE). If unique proteins are found in the cold stressed plants, these proteins will be cut from the gel, end terminally sequenced by the UC Davis Molecular Structure Facility and their sequences will be compared to others in the database. The potential effect of these proteins on *Xf* viability will be assessed as described in Objective 3. After sampling, the plants were moved and planted in the Plant Pathology field at UC Davis. Plants are currently being evaluated for symptoms to determine the most effective temperature regime for curing. This process will be repeated for the plants prepared for growth chamber and field studies in the fall/winter of 2005.

Table 5: Osmolarity and pH of xylem sap collected from grapevines in El Dorado County (Clos de Knoll Vineyard) and Yolo County (FPS) in 2004 (late February).

		El Dorado	Yolo
pH	Pinot Noir	5.37	6.35
	Cabernet Sauvignon	5.23	6.06
Osmolarity mmol/kg	Pinot Noir	55.2	58.3
	Cabernet Sauvignon	55.5	60.3

Table 6: Osmolarity and pH of xylem sap collected from grapevines in El Dorado County (Clos de Knoll Vineyard) and Yolo County (FPS) in 2005 (late March).

		El Dorado	Yolo
pH	Pinot Noir	5.87	5.79
	Cabernet Sauvignon	5.81	5.55
Osmolarity mmol/kg	Pinot Noir	34.80	37.50
	Cabernet Sauvignon	27.17	30.61

Table 7: Osmolarity and pH of xylem sap from grapevines from four locations around California- Field.

			Davis		Hopland		Fall River		Blodgett	
			1st*	2nd**	1st*	2nd**	1st*	2nd**	1st*	2nd**
pH	Pinot Noir	Control	5.81	5.79	5.96	5.73	4.94	5.97	5.88	5.23
		Inoculated	5.95	5.77	5.65	5.53	5.29	6.14	5.49	5.36
	Cabernet Sauvignon	Control	6.23	5.43	5.84	5.73	6.38	5.93	5.90	5.52
		Inoculated	6.16	5.58	5.93	5.61	6.99	5.92	6.12	5.57
Osmolarity mmol/kg	Pinot Noir	Control	44.91	37.50	42.30	54.67	59.11	35.36	67.20	69.91
		Inoculated	59.60	36.56	49.10	43.17	73.33	50.00	71.33	41.73
	Cabernet Sauvignon	Control	45.11	40.00	61.40	68.09	94.33	55.44	79.45	53.45
		Inoculated	33.33	34.80	88.30	76.00	61.00	51.00	76.33	34.64

*1st collection occurred between 2/24/05 and 3/6/05.
**2nd collection occurred between 4/15/05 and 4/22/05.

Table 8: Osmolarity and pH of xylem sap from grapevines treated with four different cold regimes- Growth Chamber.

			-5°C day; -5 °C night	+0°C day; -5°C night	+2.2°C day; -5°C night	+5°C day; -5°C night
pH	Pinot Noir	Control	5.41	5.46	5.44	5.11
		Inoculated	5.42	5.42	5.45	5.19
	Cabernet Sauvignon	Control	5.51	5.33	5.66	5.34
		Inoculated	5.54	5.66	5.59	5.72
Osmolarity mmol/kg	Pinot Noir	Control	36.5	45.3	58.5	37.6
		Inoculated	38.3	33.0	49.9	34.6
	Cabernet Sauvignon	Control	42.3	38.9	41.6	33.7
		Inoculated	45.8	45.1	37.2	25.5

Objective 3

We have assessed the effect of pH and osmolarity on the viability of *Xf* cells *in vitro* using various buffers and media such as PD3 and new chemically defined media (Leite, et al., 2004). The liquid solutions used for these viability experiments included: water, extracted xylem sap, PD3, HEPES, sodium and potassium phosphate buffers.

In order to further examine these conditions, cultures of *Xf* Stagg's Leap strain were grown at 28°C on PD3 for 11 days. Cells were scraped from the culture plates and suspended at concentrations of 1.5 x 10^7 bacteria per milliliter of liquid medium. One milliliter of the suspension was then placed into each 1.5 mL micro-centrifuge tubes and placed at various temperatures. Samples were diluted and plated out onto PD3 and allowed to grow for seven days. After seven days, colonies were counted to determine the potential effect each treatment had on the viability of *Xf* cells.

Results of these experiments indicate that *Xf* can survive at 28ºC in most media (except water). The results also indicate that *Xf* can survive at -5ºC for 8 weeks. At lower temperatures, our results were similar to those found by Feil (2002). *Xf* survived the best in HEPES and sodium phosphate buffers and the worse survival occurred in water and xylem sap at –5ºC. At –10ºC and –20ºC, *Xf* rapidly died in all liquid media tested.

Potassium phosphate buffer was used to determine the effects of pH on the survival of *Xf*. Samples were prepared like above, the cells were placed in potassium phosphate buffer at the pH levels of: 5.0, 5.4, 5.8, 6.2, 6.6 and 6.8. The cells were placed at -5ºC for up to seven days. Everyday, samples were collected and diluted and plated out onto PD3 and allowed to grow for seven days. After seven days, colonies were counted to determine the potential effect each treatment had on the viability of *Xf* cells. Results for Objective 3 are reported in the 2004 Pierce's Disease Research Symposium Proceedings.

Objective 4

Previous research has shown that herbaceous and woody plants exposed to sub-lethal cold conditions have significantly elevated levels of plant hormones, such as ABA, which induces the synthesis of a number of cold shock proteins (Bravo, et al., 1998; Thomashow, 1998). Preliminary studies, involving samples of Pinot Noir and Cabernet Sauvignon field materials collected from El Dorado County and Yolo County in February 2004, and again in March 2005, showed ABA concentrations were lower in El Dorado County, cold-exposed vines, than in vines from Yolo County. ABA concentrations were lower in Pinot Noir than Cabernet Sauvignon for both El Dorado County and Yolo County vines.

We are in the process of determining ABA concentrations of xylem sap in cold-stressed and control vines growing both in the growth chamber and in the field-grown plants in the four sites using the temperature regimes described in Objective 1.

This fall, Cabernet and Pinot vines prepared as stated in Objective 1, will be sprayed with 100µM solutions of ABA, a concentration that elicited cold-shock proteins at 23°C in winter wheat (Kuwabara, et. al 2002). Additional concentrations up to 500µM may also be evaluated if no response is noted at 100µM. The pH and osmolarity of xylem sap from the treated vines will be determined as described above. The concentration of ABA in the sap will be determined using a commercially available immunoassay that has a sensitivity of 0.02-0.5 picomole/0.1 ml (Plant Growth Regulator Immunoassay Detection Kits, Sigma Chemical Co.). Preliminary work has shown that ABA concentrations in grapevine xylem sap are detectable using this kit. Xylem sap proteins will be collected, concentrated and analyzed by 1- and 2-dimensional PAGE as previously described. Unique proteins expressed in ABA-treated vines will be removed from the gels and end terminally sequenced and analyzed as previously described. We will also determine the pH, osmolarity and protein profiles of xylem sap from ABA-treated vs. non-treated vines and assess the potential of this sap for anti-*Xf* activity.

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INTERACTION BETWEEN *XYLELLA FASTIDIOSA* AND *CURTOBACTERIUM FLACCUMFACIENS*, AN ENDOPHYTIC BACTERIUM

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ABSTRACT

Xylella fastidiosa (*Xf*) is a fastidious gram-negative, xylem-limited bacterium that causes diseases in many crops of economic importance, such as grape (Pierce's disease), almond, peach, coffee, plum and citrus. *Xf* can infect all known *Citrus sinensis* cultivars and causes Citrus variegated chlorosis (CVC). One major endophyte isolated from CVC-asymptomatic plants is the bacterium *Curtobacterium flaccumfaciens* (*Cf*). *Catharanthus roseus* (*Cr*) plants were inoculated together with *Cf* and *Xf* and in a similar experiment, plants were inoculated alone with *Xf* and *Cf*. Three phytopathology parameters, including number of flowers, height of plants, and disease symptoms were evaluated. Primers for *Cf* were designed to detect this endophytic bacterium in plant tissue when inoculated with *Xf*. These primers were able to detect *Cf* in the presence of *Xf* after inoculation in *Cr*. *In planta* interaction studies where *Cf* was inoculated together with *Xf* showed that there was an inhibition of disease symptoms caused by *Xf*.

INTRODUCTION

Endophytes are microorganisms that do not visibly harm the host plant but can be isolated from surface-disinfected plant tissue or the inner parts of plants (Hallmann et al. 1997). Since they colonize an ecological niche similar to that of phytopathogens, endophtytes are candidates for biocontrol agents (Hallmann et al. 1997). Members of the genus *Curtobacterium* have been isolated as endophytic bacteria from many plants, including red clover (Sturz & Christie, 1998), rice (Elbeltagy et al. 2000), potato (Sturz & Matheson, 1996), yam (Tor et al. 1992), citrus (Araújo et al. 2002; Lacava et al. 2004), and are associated with control of plant diseases in tobacco (Park & Kloepper, 2000), cucumber (Raupach & Kloepper, 2000) and potato (Sturz & Matheson, 1996); and plant growth-promotion of red clover (Sturz et al. 1997) or interacting with other bacteria in plant growth-promotion (Bent & Chanway, 1998).

CVC is a disease of sweet orange trees (*Citrus sinensis* L.) caused by one strain of the xylem-limited bacterium *Xf* (Hartung et al. 1994). *Xf* is transmitted by xylem-feeding sharpshooter leafhoppers (*Homoptera: Cicadellidae, Cicadellinae*; Roberto et al., 1996; Brlansky et al., 2002) or through seeds (Li et al., 2003). In Brazil, CVC is responsible for losses to the citrus industry of US $ 100 million per year (Coletta-Filho et al., 2001). In spite of the fact that *Xf* was the first plant pathogen to have its genome completely sequenced (Simpson et al. 2000), much remains to be learned about its pathogenesis, biology and ecology.

Araújo et al. (2002) and Lacava et al. (2004) demonstrated that *Cf* is isolated more frequently from CVC-asymptomatic than CVC-symptomatic orange and tangerine plants. Also, Lacava et al. (2004) found, through the use of *in vitro* interaction experiments that the growth of *Xf* could be inhibited by the presence of endophytic *Cf*.

OBJECTIVES

1. Evaluate, *in planta*, the interaction between *Xf* and *Cf* and the potential use of this endophytic bacterium in biological control

RESULTS

Sixty days after the inoculation of *Cr* seedlings, *Cf* was detected by PCR using primers CFC1 and CFC2. *Xf* was also specifically detected using primers 271-int and 272-int on extracts of *Cr* inoculated with *Xf*. In seedlings simultaneously inoculated (doubly-inoculated), *Cf* was detected by PCR with primers CFC1 and CFC2 and *Xf* with primers 271-int and 272-int respectively.

The first parameter analyzed to check the effects of inoculation of *Xf* and *Cf* was the number of flowers. Plants inoculated with sterile PW medium (negative control) and plants inoculated with *Cf* did not demonstrate a statistically significant difference (< P 0.05) in the number of flowers. Plants inoculated with *Xf* alone had a reduced number of flowers (< P 0.05)

during the same period (Figure 1). However, plants inoculated with both *Xf* and *Cf* demonstrated a number of flowers similar (< P 0.05) to those inoculated with PW media, *Cf* and *Xf* (Figure 1).

In the second parameter analyzed, the height of plants were statistically similar in plants inoculated with PW medium and plants inoculated with *Xf* and *Cf* (< P 0.05) (Figure 2), but plants inoculated with *Xf* demonstrated reduced height after 60 days (Figure 2).

After sixty days, plants inoculated with sterile PW medium (negative control) did not demonstrate symptoms of disease and neither did plants inoculated with C*f* or with both *Xf* and *Cf* (double-inoculation). However, plants inoculated with just *Xf* demonstrated characteristic symptoms of disease (Figures 3 and 4).

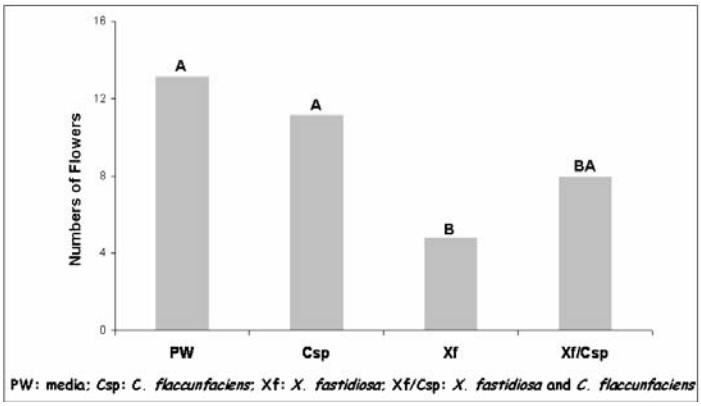

Figure 1. Interaction between *Cf* and *Xf* inside of host plant, *Cr*, after two months. The phytopathology parameter used to evaluate the interaction was number of flowers. Different letters on bars show statistical difference by Tukey's test at 5% of significance.

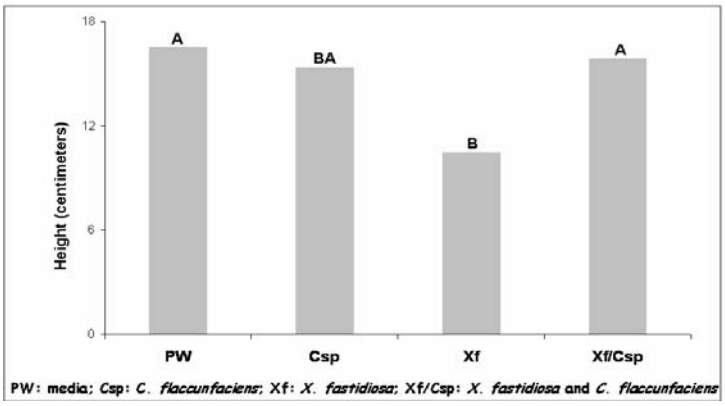

Figure 2. Interaction between *Cf* and *Xf* inside of host plant, *Cr*, after two months. The phytopathology parameter used to evaluate the interaction was height. Different letters on bars show statistical difference by Tukey's test at 5% of significance

Figure 3. *Cr* plants two months after inoculation with *Xf* (right) in comparison with *Xf* and *Cf* inoculated together doubly-inoculated (left).

Figure 4. *Cr* leaves two months after inoculation with *Xf* (left) and doubly-inoculated with *Xf* and *Cf* (right) in inoculated in plant.

CONCLUSIONS

The endophytic bacterium *Cf* was detected in extracts of *Cr* 60 days after inoculation using the primer pair CFC1/CFC2 in a PCR assay. In a similar experiment, where both *Cf* and *Xf* were inoculated into *Cr*, it was possible to detect both the endophyte and the pathogen using PCR. These data demonstrate that *Cf* has the ability to colonize plant tissue in presence of *Xf*. This is an important point to consider when evaluating this endophyte as a potential biocontrol agent for CVC.

The parameters measured to check the potential use of *Cf* against *Xf* include number of flowers, height, and disease symptoms. This study suggested that this endophyte was able to reduce the effect of the colonization of *Xf*. In plants inoculated with *Xf* and *Cf,* symptom remission probably occurred compared with plants inoculated just with *Xf*.

Recently, an interaction between *Cf* and *Xf* was strongly indicated (Araújo et al., 2002; Lacava et al., 2004). These authors suggested this interaction based in the frequency of isolation of *Cf* and in interaction experiments *in vitro* using both *Xf* and *Cf*. This article describes how *Cf* can reduce symptoms caused by *Xf in planta* when both the phytopathogen and the endophytic bacterium colonize the same plant.

This work described the effect of a possible interaction of *Cf* and *Xf in planta* under controlled conditions and the results reinforce the idea that endophytic bacteria, that colonize a similar niche as does *Xf*, could contribute to the reduction of the symptoms in the field.

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SOLUBLE FORMS OF AN ANTI-*XYLELLA* ANTIBODY AND STRAINS OF *ALCALIGENES XYLOSOXIDANS DENITRIFICANS* CAPABLE OF SECRETING THEM

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ABSTRACT
Several methods were used to create soluble forms of a single chain antibody (scFv S1) that binds to the surface of the grape strains of *Xylella fastidiosa* (*Xf*). S1 fused to a *pelB* leader and secreted from *E. coli*. These forms were not secreted correctly and could not bind *Xf* in an ELISA. Maltose binding protein fusions of S1 were soluble and could be used to detect *Xf* in an ELISA. We also successfully secreted S1 from *Alcaligenes xylosoxidans denitrificans* (*Axd*) using a leader sequence that directed S1 to the periplasmic space. Strains of *Axd* that secrete anti-*Xylella* factors are being developed for use in a strategy to prevent the spread of *Xf*.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) is the principal vector of the xylem-limited bacterium *Xylella fastidiosa (Xf)*, which causes Pierce's disease (PD) in grapes. Limiting the spread of this pathogen by rendering GWSS incapable of pathogen transmission (paratransgenesis) is a promising method of pathogen control. Paratransgenesis seeks to modify the phenotype of an organism indirectly by modifying its symbiotic bacteria to confer vector-incompetence

Paratransgenic approaches to disrupt pathogen infection of humans are being developed by several groups. These include interference with the ability of triatomid bugs to transmit pathogens causing Chagas' disease (Beard et al., 2001), interference with HIV attachment to its target cells in the reproductive tracts of humans (Chang et al., 2003; Rao et al., 2005), and the elimination of persistent *Candida* infections from biofilms in chronically infected patients (Beninati et al., 2000). Paratransgenesis has also been applied to deliver cytokines mammalian guts to relieve colitis (Steidler et al., 2000; Steidler, 2001). Thus, the method has wide applicability.

Alcaligenes xylosoxidans denitrificans (Axd) is Gram negative, beta proteobacterial species that can colonize the GWSS foregut and cibarium, as well as various plant tissues, including xylem. It is non-pathogenic in insects, plants and healthy humans. Given these characteristics, *Axd* has become the focus of our paratransgenesis efforts to control PD in grapes. Over the past several years we developed the technology to stably modify *Axd* by inserting genes into its chromosome, have developed methods to suppress horizontal gene transfer, and have isolated a single chain antibody (scFv) that recognized an epitope on the surface of the PD strain of *Xf*. (Bextine et al., 2004). We are currently engaged in combining these systems in order to produce strains of *Axd* that are suitable for environmental release in a practical strategy symbiotic control strategy for PD.

We report here the evaluation of various S1 constructs for solubility and the construction of a prototype *Axd* strain capable of secreting S1.

OBJECTIVES
1. To create soluble and functional forms of the S1 single chain antibody.
2. To construct strains of Axd capable of secreting scFvs.

RESULTS
Objective 1: Soluble forms of the S1 scF.
We expressed a soluble form of the S1 scFv in two ways. S1 was expressed from a construct carrying a *pelB* leader sequence which targets the protein to the periplasm of the cell, from which it can "leak" out into the growth medium and be collected. Several strains of *E. coli* were used for this test. We also fused the S1 sequence to *E. coli* maltose binding protein and purified the fusion protein using affinity chromatography. S1 proteins expressed in these two ways were assayed in a western blot to see if they could be detected at all and were also used in an ELISA to determine whether or not they could still bind to the surface of *Xf*. The results of these assays are shown in Table 1.

Table 1. Constructs, expression, and ELISA details for soluble S1 anti-*Xylella* scFv.

Protein expression Species / Strain	**S1 construct**	**Detectable in Western?**	**Detectable in *Xylella* ELISA?**
E. coli Top10F'	pAM5: Plac-driven expression of S1.	Yes-Strong expression[1]	No
E. coli HB2151	pAM5: Plac-driven expression of S1.	Yes- Weak expression	No
E. coli TB1	pAM5: Plac-driven expression of S1.	Yes- Weak expression	No
E. coli Top10F'	pAM62: Plac-driven expression of MBP[2] - full-length S1.	Yes (purified protein)	Yes
E. coli Top10F'	pAM63: Plac-driven expression of MBP-S1 lacing periplasmic S1 targeting sequence.	Yes (purified protein)	Yes

[1]. The non-pMAL constructs used in this table are meant to secrete a soluble scFv into the supernatant of the culture by leakage from the periplasmic space.
[2]. MBP = maltose binding protein.

As can be seen in Table 1, there was a strong strain-specific effect on the amount of expression of S1. Furthermore, in each case, the proteins secreted from *E. coli* were of the wrong size (see below) and did not bind to the surface of *Xylella* in an ELISA. On the other hand, purified maltose binding protein-S1 fusion proteins were easily detected on westerns and retained the S1 binding activity which is present when S1 is fused to the gIII protein of M13 phage and is present as part of a viral particle.

Objective 2: Secretion of an S1 scFv construct from Axd

Secretion from Gram negative bacteria is complicated by the fact that these species have two membranes that a protein must cross before appearing outside the cell. Gram negatives contain at least six identified types of secretion systems. Unfortunately, these systems are unpredictable when expressed heterologously. In other words, there is no "one-size-fits-all" system that can be used in all species. Periplasmic targeting, however, can be achieved easily by using leader sequences that are functional in a broad range of organisms. We used the *pelB* leader from *Erwinia caratovora* to target S1 to the periplasm of *E. coli* and *Axd*. Constructs were made as single-copy insertions into the chromosome delivered on a *mariner* transposon. Cultures of these strains were allowed to grow overnight for *E. coli* and for two days for *Axd*. Western analysis was used to detect the S1 in the medium. The results are shown in Figure 1.

As noted above, *E. coli* expressed S1 incorrectly when fused with a *pelB* leader for periplasmic expression. Material of ca. 40kD, from the media and the pellet could be detected in the western. Furthermore, higher molecular weight material of ca. 70 kD could be detected only in the pellet. Media in which S1-*Axd* strains were grown, however, secreted a protein of the correct size for monomeric S1 (ca. 26 kD). In overloaded lanes of pellet (= cell lysate) material, a product of 70 kD could also be detected as it was in *E. coli*. These results indicate that periplasmic targeting and "leakage" from the periplasmic space can be used as a kind of secretion system in *Axd*. The difference in expression and secretion of S1 in two different Gram-negative bacterial species clearly indicates that species-specific factors are at work and need to be carefully evaluated when constructing strains for use in symbiotic control.

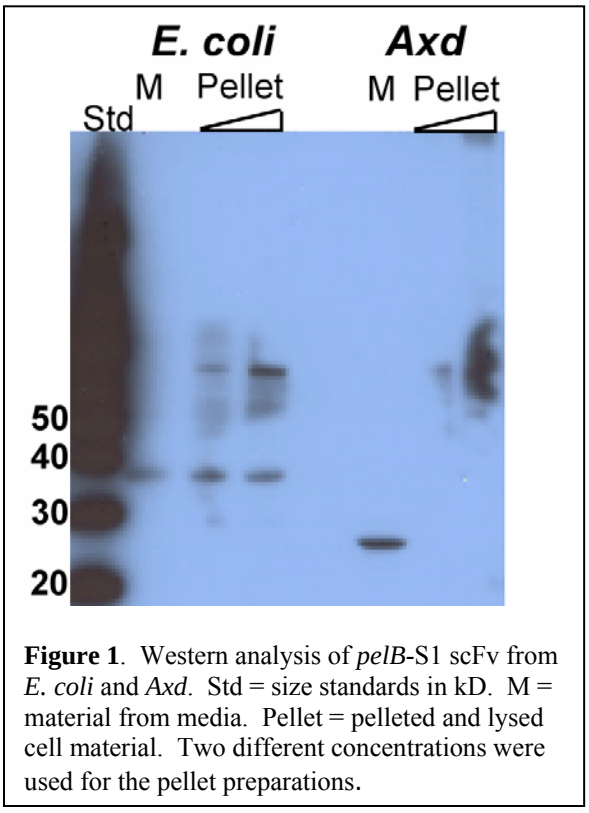

Figure 1. Western analysis of *pelB*-S1 scFv from *E. coli* and *Axd*. Std = size standards in kD. M = material from media. Pellet = pelleted and lysed cell material. Two different concentrations were used for the pellet preparations.

CONCLUSIONS

Paratransgenesis to control PD in grapevine requires that several conditions be met. First, a suitable microorganism must be found. Second, anti-*Xylella* factors must be isolated. Third, strains of the microorganism must be constructed that can deliver the anti-*Xylella* factor appropriately and in an environmentally-safe way. We previously isolated an anti-*Xylella* single chain antibody by panning a scFv phage library. The phage antibody reacts strongly only with grape strains of *Xf*. We converted the scFv from phage-form to soluble form by targeting the scFv to the periplasmic space without the phage gIII protein. In *E. coli*, this results in an incorrectly produced protein that lacks the ability to bind *Xf*. Expression of S1 as a maltose binding protein fusion results in a soluble protein that retains *Xf* binding ability. We thus conclude that it is possible to convert the phage antibody to a soluble form; however we will need to evaluate different constructs for proper binding and secretion behavior. Finally, we were able to successfully secrete S1 from transgenic strains of *Axd* by using the very simple method of targeting the protein to

the periplasmic space. Protein collected from the medium after two days was of the correct size. We conclude that secreting strains of *Axd* are possible routes to the creation of usable bacterial strains targeting *Xf* in a paratransgenesis approach to controlling PD.

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ECOLOGICAL AND GENETIC CHARACTERISTICS ASSOCIATED WITH *ALCALIGENES XYLOSOXIDANS DENITRIFICANS*

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ABSTRACT
A bacterium isolated from *Homalodisca coagulata* Say, found also to inhabit xylem of citrus and grape (Lauzon et al. unpubl.), shows potential for use in Symbiotic Control strategies against *Xylella fastidiosa* (*Xf*), the causal organism of Pierce's disease (PD). The biology and "behavior" of the bacterium, identified as *Alcaligenes xylosoxidans denitrificans* (*Axd*), is under study to gather information that can be used to assess its efficacy and risk of use in the field. Real Time-Polymerase Chain Reaction (RT-PCR) was used as a semi-quantitative means of monitoring *Axd* growth in lake water under semi-natural conditions. *Axd* grew better in autoclaved lake water than in lake water that contained indigenous microbial populations. *Axd* growth was also monitored in soil and on leaf surfaces under semi-natural conditions using microbiological and molecular techniques. *Axd* was not retrieved from soils containing indigenous microbial populations unless the soil was autoclaved. *Axd* was retrieved from leaf surfaces from citrus, strawberry, sage, and basil. We are currently examining the effect of introducing *Axd* to citrus leaf microbial communities using denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism. We have also monitored transfer and uptake of two plasmid vectors, DsRed (pIRES-DsRed Express, Invitrogen) and pTZ18r (Amersham Biotech). Transformation attempts included both chemical and electroporation protocols. *E. coli* was used as a control. In both cases, *Axd* resisted transformation while *E. coli* was successfully transformed. In addition, *Axd* was screened for the presence of endogenous plasmids. A strain of *E. coli* containing a single copy plasmid was used as a control. Our data suggest that *Axd* does not contain any endogenous plasmids from 5-150 kb. We are currently examining horizontal gene transfer potential between *Axd* and strains of *E. coli* and *Shigella* sp. that carry fluorescent and antibiotic-marked endogenous plasmids. Horizontal gene transfer is yet one factor used to assess harm and risk. It has been inferred that *Axd* is a potential new human pathogen based in part on its association with *Pseudomonas aeruginosa* (*P. aeruginosa*) infections in Cystic Fibrosis patients. We used RT-PCR to compare the growth of *Axd* and *P. aeruginosa* individually and in co-culture. We found that *Axd* and *P. aeruginosa* significantly affect each others' growth. Our data suggest that *Axd* has the potential to mitigate harm associated with *P. aeruginosa* infections.

INTRODUCTION
Axd is a potential candidate for Symbiotic Control of PD. Symbiotic Control strategies engage beneficial microorganisms to control pathogenic microorganisms. This includes the use of a symbiont to deliver an antimicrobial product (i.e. Beard et al. 2002). The use of *Axd* in the management or control of PD requires that *Axd* remain in ecosystems for limited but effective periods of time and cause minimal and reversible, or no disruption to a host or ecosystem. To begin to assess efficacy and risk associated with the use of *Axd* in the field, we conducted studies aimed to monitor the fate of *Axd* in soil, water, and plant ecosystems under semi-natural conditions. We also examined the potential of *Axd* to engage in horizontal gene transfer. Finally, because *Axd* has been reported as a possible new human pathogen based in part on its association with *P. aeruginosa* infections in Cystic Fibrosis, we examined growth of both bacterial species alone and in co-culture using Real Time PCR.

OBJECTIVES
1. Determine if *Axd* possesses plasmids of high, medium-low, and very low copy number.
2. Determine if *Axd* participates in horizontal gene transfer, namely transformation.
3. Assess the impact of adding GM *Axd* to microbial communities present in various ecosystems.
4. Assess the competitive vigor of *Axd* when grown in co-culture with *P. aeruginosa*.

RESULTS

Objective 1: Determine if *Axd* possesses plasmids

No plasmids were found in *Axd* (Figures 1-3). *E. coli* containing a single copy plasmid/cell was used in all detection preparations as a positive control. High, medium-low and very low copy number procedures were employed including the use of Field Inversion Gel Electrophoresis for improved separation and resolution of DNA (i.e. discerning between a mix of genomic and plasmid DNA). *Axd* does not possess plasmids ranging in size from 5 to 150 kb.

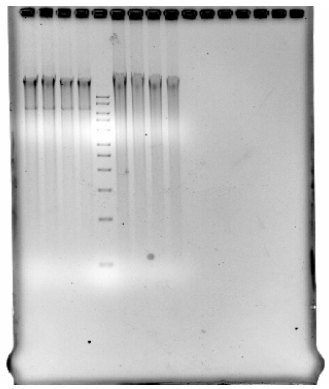


Figure 1: 5 ml and 10 ml culture volumes of *Axd* used for high copy plasmid preparation. A 5 ml– 10 ml culture volume is generally enough to detect a high copy plasmid in bacteria. No DNA bands are present in the lanes 2-5. Lane 1- 10 kb DNA marker. The highest band is 10 kb and the lowest is 1 kb. Lane 2,3 –5 ml culture volume of *Axd* plasmid prep. Lane 4,5 -10 ml culture volume of *Axd* plasmid prep. Note: 5ml and 10 ml refer to the culture volumes that were subjected to plasmid prep and not the amounts that were actually loaded onto the gel.

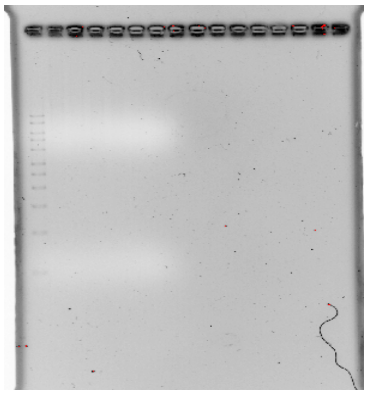


Figure 2: 500 ml culture volume of *E. coli* containing pBeloBac plasmid DNA and *Axd* were used for medium-low plasmid prep. A 500 ml culture volume is generally enough to detect a medium copy plasmid (30-300 copies/cell), low copy (5 – 30 copies/cell) and very low copy (less than 5 copies/cell) of plasmid DNA. Lanes 1-4 –500 ml culture volume of *E. coli* containing pBeloBac plasmid DNA. Lane 5- 10 kb DNA marker with the highest band at 10 kb, and the lowest at 1 kb. The sizes from top to bottom are 10 kb, 8 kb, 6 kb , 5 kb , 4kb, 3 kb, 2.5 kb, 2 kb, 1.5 kb and 1 kb. Lane 6 -9 –500 ml culture volume of *Axd* plasmid prep. No bands were detected below the 10 kb marker.

Figure 3: A 2 liter culture volume of *Axd* used for a very low plasmid prep. A 500 ml culture volume is generally enough to detect a very low copy plasmid such as a single copy plasmid/cell, however, we also performed a 2 liter plasmid prep for *Axd* to be thorough. No bands were observed in lanes 5-8. Lanes 1- 3 – empty. Lane 4- 10 kb DNA marker. The highest band is 10 kb, the lowest is 1 kb. Lanes 5-8 –2 liter culture volume A*xd* plasmid prep. Some bands can be seen above the 10 kb marker size in Lanes 5-7, however, we don't know if this is plasmid DNA or sheared genomic DNA. Therefore, the plasmid prep sample was subjected to FIGE – field inversion gel electrophoresis which helps separate distinct plasmid DNA from smears of genomic DNA much better than separation on an agarose gel. We conclude that *Axd* does not have plasmids of sizes ranging from 5- 150 kb.

Objective 2: Determine if *Axd* participates in horizontal gene transfer, namely transformation

Axd was subjected to chemical ($CaCl_2$) and electroporation techniques in attempts to transform *Axd* with two different plasmids. Plasmids pIRES2-DsRedExpress (Invitrogen) and pTZ18r (Amersham Biotech) were used for all procedures. A strain of *E. coli* amenable to transformation was used as a control. Results show that *Axd* was not easily amenable to transformation. There is a slight possibility that promoters do not work in *Axd* for these plasmids, although this is doubtful. Similar results have been found in the Lampe laboratory (personal communication).

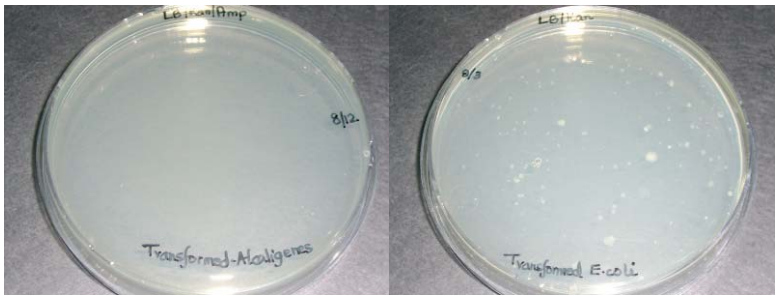


Figure 2. *Axd* plated onto Luria Bertani (left) agar post-transformation protocols. Notice that no growth occurred for *Axd* on a medium that would support the growth of a transformed strain. *E. coli* were transformed (right), grew well, and thus, procedures were conducted properly.

Objective 3: Assess the impact of adding GM *Axd* to microbial communities of various ecosystems
GM *Axd* containing EGFP fluorescent gene was used in all studies. GM *Axd* was applied to leaf surfaces of three different hosts and detected using microbiological and molecular techniques. GM *Axd* was also added to autoclaved and non autoclaved lake water and detected over time using molecular means (Figure 4). Real Time PCR was used to monitor the growth of GM *Axd* over time using primers for EGFP and 16 S rRNA genes. Data reveal poor growth of GM *Axd* in lake water (complete data not shown).

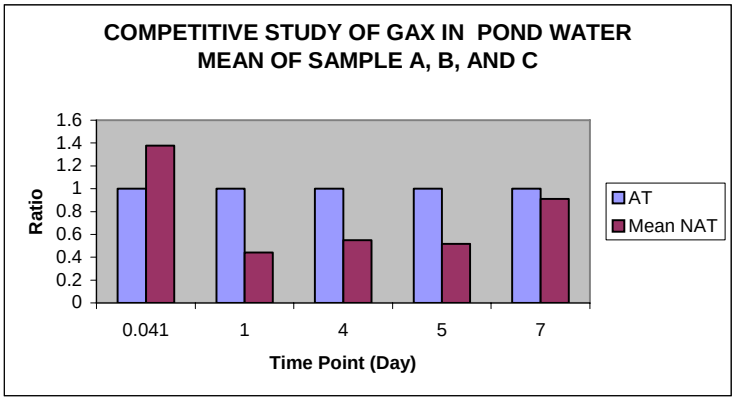
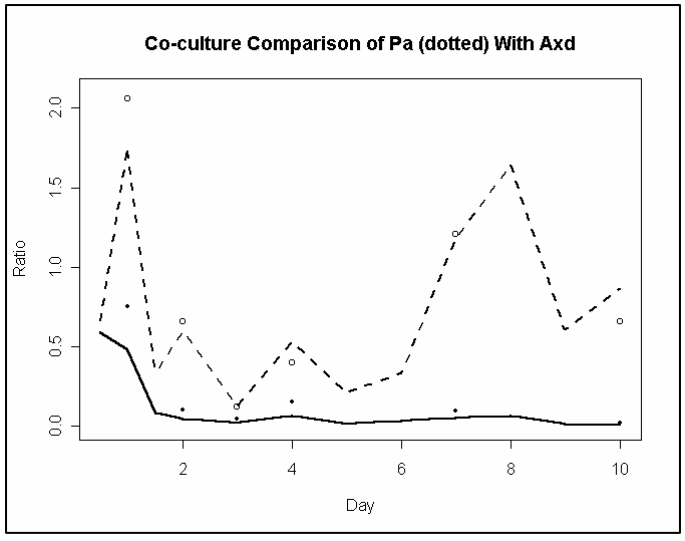


Figure 4.

Studies have recently commenced for determining the impact of inoculating citrus and grape leaf surfaces with *Axd* on community structure. Meyer Lemon leaves were inoculated with both 10 and 100 µL volumes of an 18 h culture of GM *Axd* to confirm detection of *Axd* using both primers for 16S rDNA and primers for EGFP. Detection was successful (data not shown).



Objective 4: Assess the competitive vigor of *Axd* when grown in co-culture with P. aeruginosa
Real Time PCR revealed that when grown together in liquid medium, *P. aeruginosa* and *Axd* significantly affect (decrease) each others' growth. Ratios were generated for mixed samples in relation to individual growth values over time and ranked. Treatments (batches) were replicated and two trials were conducted. No differences were found statistically in batches. ANOVA on ranks were used because residuals in ANOVA on ratios indicated non-normal data. ANOVA ranks showed that *P. aeruginosa* grew better in the presence of *Axd* than did *Axd* in the presence of *P. aeruginosa*; however, *P. aeruginosa* growth was less than that in pure culture. After 24 h, the presence of *Axd* significantly decreases the growth of *P. aeruginosa* but after 7 days of interactions, P. *aeruginosa* cell numbers increase significantly.

CONCLUSIONS
We have found that *Axd* is not easily transformed and does not contain plasmids ranging in size from 5-150 kb. *Axd* also does not establish well in established ecosystems but likely remains in an environment long enough to exert an effect on *Xf* (Bextine et al. 2005). When grown in the presence of the human pathogen, *P. aeruginosa*, *Axd* growth is less than that seen in pure culture. In addition, the presence of *Axd* decreases growth of *P. aeruginosa*. These data question recent inferences that *Axd* is a nosocomial or harmful bacterium to humans. While our findings provide important information regarding risk assessment and use of *Axd* in the field, further studies are necessary, including those that monitor the fate of *Axd* through successive field seasons and changing environmental and plant physiological conditions. To date, no concrete evidence exists that show *Axd* as a harmful bacterium to any ecosystem or host.

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MANAGEMENT OF PIERCE'S DISEASE OF GRAPE BY INTERFERING WITH CELL-CELL COMMUNICATION IN *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted October 2004 to October 2005.

ABSTRACT
Xylella fastidiosa (*Xf*) has homologs of the cell-cell signaling genes found in the important plant pathogen *Xanthomonas campestris* pathovar *campestris* (*Xcc*) and produces a similar alpha,beta unsaturated fatty acid signal molecule called DSF that coordinates gene expression. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease (PD). The *rpfF* gene is necessary and sufficient for DSF signal synthesis and *rpfF* mutants of *Xf* are hypervirulent and non-transmissible. Lack of transmissibility was linked to an inability of the *rpfF* mutant to form a biofilm in the insect foregut; while taken up by insects, the mutant strain is not retained. *Xf* strains that overproduce DSF produce disease symptoms in grape, but only at the site of inoculation and the cells do not move within the plant as do wild-type strains. Thus elevating DSF levels in plants should reduce movement of *Xf* in the plant and also reduce the likelihood of transmission by sharpshooters. We identified bacterial strains that can interfere with *Xf* signaling both by producing large amounts of DSF, by degrading DSF, or by in some way interfering with recognition of DSF. When co-inoculated into grape with *Xf*, both DSF-producing strains and DSF-degrading strains greatly reduced the incidence and severity of disease in grape; DSF-producing strains consistently were the most effective in reducing disease. Disease was also reduced when some of these strains were simply sprayed onto grape before inoculation with *Xf,* indicating that they can alter behavior of the pathogen even when not co-inoculated. To verify that disease control is due to DSF interference, we have constructed mutants of these strains that disrupt the ability of these strains to produce or degrade DSF and show that the mutants are deficient in PD control. Both mutants unable to produce DSF as well as mutants deficient in degradation of DSF exhibited less ability to control PD when co-inoculated with *Xf*, suggesting that altering DSF abundance within the plant was a major factor contributing to disease control by these DSF-interfering strains. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf* we have transformed grape with the *rpfF* gene of *Xf* to enable DSF production in planta. Transgenic plants are being assayed for DSF production and susceptibility to *Xf* infection. The bacterial genes required for DSF degradation have been cloned and identified in antagonist Pseudomonas strain G, enabling their exploitation for disease control by over-expression in various bacterial endophytes of grape as well as by expression within plants themselves. Non-endophytic bacterial species were also established in high numbers inside grape leaves and petioles following spray application to plants with a high concentration of a silicon-based surfactant with a low surface tension. PD was reduced in plants after topical application of a DSF-producing strain of *Erwinia herbicola* (*E. herbicola)*.

INTRODUCTION
Endophytic bacteria such as *Xf* colonize the internal tissues of the host, forming a biofilm inside the plant. A key determinant of success for an endophyte is the ability to move within the plant. We expect activities required for movement to be most successful when carried out by a community of cells since individual cells may be incapable of completing the feat on their own. Cells assess the size of their local population via cell-cell communication and coordinately regulate the expression of genes required for such processes. Our study aims to determine the role of cell-cell communication in *Xf* in colonization and pathogenicity in grapevines and transmission by the insect vector. *Xf* shares sequence similarity with the plant pathogen *Xcc* (1). In *Xcc,* expression of pathogenicity genes is controlled by the *rpf* system of cell-cell communication, enabling a population of cells to launch a pathogenic attack in a coordinated manner (1). Two of the *rpf* proteins, *rpfB* and *rpfF*, work to produce a diffusible signal factor (DSF) (2) which has recently been described as an alpha,beta unsaturated fatty acid (3) (Figure 1).

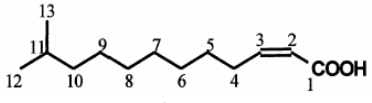

Figure 1

As the population grows, the local concentration of DSF increases. Other *rpf* proteins are thought to sense the increase in DSF concentration and transduce a signal, resulting in expression of pathogenicity factors. The *Xf* genome not only contains homologs of the *rpf* genes most essential for cell-cell signaling in *Xcc*, but also exhibits striking colinearity in the arrangement of these genes on the chromosome. We now have shown that *Xf* makes a molecule that is recognized by *Xcc* but

probably slightly different than the DSF of *Xcc*. Based on our knowledge of density-dependent gene regulation in other species, we predict the targets of *rpf* regulation would be genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel. Similarly, we would expect the density-dependent genes to be expressed during the time when a population of *Xf* is ready to move into un-colonized areas.

Other organisms can apparently interfere with the density-dependent behaviors of *Xf*. Several recent studies indicate that other organisms can disrupt or manipulate the cell-cell signaling system of bacteria. Our preliminary work revealed that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf*, but until this study we did not know of the manner in which the interaction occurred nor whether such strains had the potential to affect the virulence of *Xf* in grape. In this period we have extensively investigated both the role of DSF-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, initiated genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control.

OBJECTIVES

1. Identify bacteria that interfere with DSF-mediated cell-cell signaling in *Xf*, and conduct pathogenicity tests on grapevines colonized by DSF-interfering bacteria to determine potential for PD control
2. Isolation of mutant strains of DSF-degrading and DSF-producing bacteria that can no longer interfere in cell-cell signaling to verify that disease control is linked to cell-cell signal interference
3. Molecular identification of genes conferring DSF-degrading activity
4. Engineer the grapevine endophytes *Alcaligenes xylosoxidans denitrificans* and *Agrobacterium vitis* to express genes conferring DSF-degradation and DSF-synthesis activities and test whether the resulting transgenic endophytes are capable of disease control
5. Creation of grapevines expressing genes conferring DSF-degradation and DSF-synthesis activity to test for PD resistance
6. Evaluate topical application of DSF-degrading and DSF-producing bacteria with penetrating surfactants for PD control

RESULTS

We have isolated a variety of bacteria from grapevine vineyards affected by PD as well as tomato and cruciferous crop plants infected with the signal-producing pathogens *Xanthomonas campestris* pv. *vessicatoria* and *Xcc*, respectively and tested them for their ability to interfere with cell-cell signaling in *Xf* in an assay using the signal-sensing strain described above. We found several strains that negatively affected signaling in *Xcc* while several strains were found to produce DSF. By adding purified DSF to either cell-free extracts of the strains with a negative influence on signaling or to whole cells we found that at least two mechanisms of interference with signaling could be observed. Some strains such as strains C,E,G, H, and J are able to degrade DSF while other inhibitor strains did not do so, and apparently have another means of interfering with DSF perception by *Xcc*. The several strains that produced DSF were all identified as *Xanthomonas* species. We sequenced the 16S rRNA gene from these strains to determine their species identity.

To test the ability of bacteria that alter *Xf* signaling to alter the process of disease in plants, we co-inoculated grapevines with *Xf* and strains that either inhibit or activate cell-cell signaling in greenhouse studies. The incidence of PD was greatly reduced by all of the signaling interfering strains that we tested (Figures 2 and 3). As we had expected, DSF-producing strains generally reduced disease severity more than did strains that interfered with signaling in *Xf*. These results were highly repeatable, having been observed in five separate experiments. We find these results to be very exciting in that they suggest that alteration of signal molecules within plants can have a profound effect on the disease process.

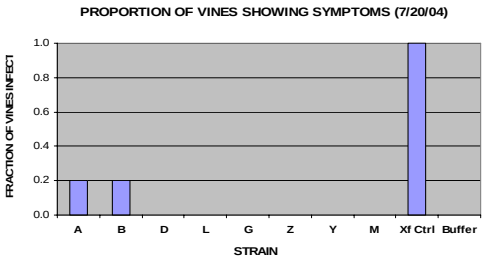

Figure 2. Incidence of PD in grape co-inoculated with *Xf* Temecula and various DSF-producing and degrading bacterial strains.

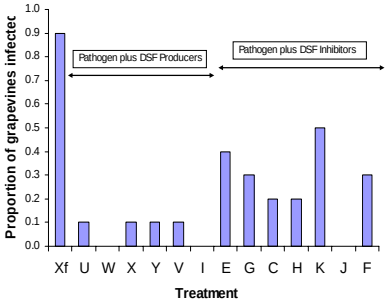

Figure 3. Incidence of PD in grape co-inoculated with *Xf* Temecula and various DSF-producing and degrading bacterial strains.

We also have been able to provide disease control by topical inoculation of DSF-producing bacteria such as DSF-producing strains X and 8004 to the foliage of plants where they colonize and presumably produce DSF as well as by pre-treatment of plants by injection of these antagonists before inoculation with *Xf* (Figure 4).

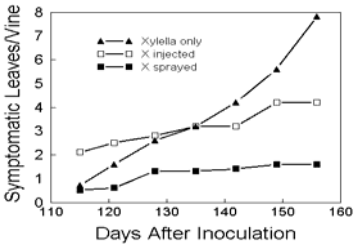

Figure 4. Severity of PD in grape co-inoculated with DSF-producing strain X or sprayed with this strain before inoculation with Temecula compared to plants inoculated only with Temecula.

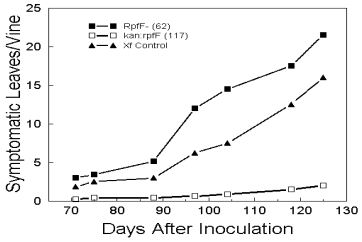

Figure 5. Severity of PD in grape co-inoculated with a DSF over-producing strain of *Xf* or with an *rpfF* mutant compared to plants inoculated with Temecula.

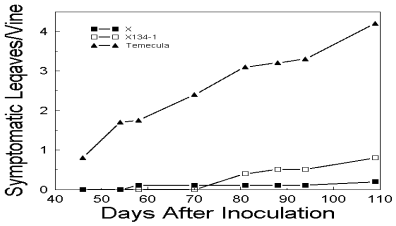

Figure 6. Severity of PD in grape co-inoculated with DSF-producing strain X or a mutant of X that does not produce DSF compared to plants inoculated only with Temecula.

To determine the extent to which altered DSF abundance in plants would alter the progress of PD we also made mutants of *Xf* that were either blocked in DSF production or over-expressed DSF. A strain of *Xf* Temecula in which the *rpfF* gene, which is required for production of the signal in *Xcc*, is knocked out was constructed using exchange of the wild-type allele for a deleted copy carrying an antibiotic resistance gene on a suicide plasmid. The *rpfF* mutant of *Xf* does not make DSF as determined using previously constructed "signal-sensing" strains of *Xcc* to determine DSF production by *Xf* and other bacterial strains. We also over-expressed DSF by introducing the *rpfF* gene driven by a constitutive *kan* promoter into the genome of *Xf*. This strain produced much higher levels of DSF than the parental strain. The strains altered in DSF production were tested for their ability to infect and move within host plants and to cause PD symptoms. The *rpfF* gene appears to play a role in modulating disease progress because the timing and severity of symptom development are greatly exacerbated in grapevines infected with *rpf*-deficient mutants when compared to the wild type (Figure 5). In contrast, the *Xf* strain that overproduced DSF caused disease symptoms in grape, but only at the site of inoculation. The mutant cells did not move within the plant as did wild-type strains (Figure 5). These results all support our model that DSF regulates genes required for movement of *Xf* from colonized vessels. We hypothesize that *rpfF*-deficient mutants may be causing increased vessel blockage in the grapevine, leading to increased symptom expression.

To establish a rigorous connection between DSF production and disease control, we have constructed mutant strains of those DSF-producing bacteria that perform best in the disease control assays that no longer could produce DSF. These mutants were then compared to their parent strains in disease control assays. We also made mutants of DSF-degrading strains that no longer could degrade DSF. We expected that if DSF interference can provide disease control, then strains no longer able to interfere with DSF signaling will also no longer be able to control disease. All mutants unable to produce DSF were diminished in ability to reduce PD when co-inoculated with *Xf* compared to their DSF-producing wild-type strain (Figures 7-8).

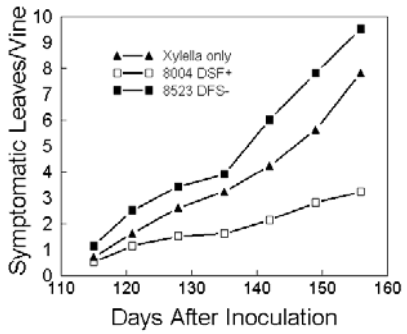

Figure 7. Severity of PD on grape co-inoculated with an *Xcc* DSF-producing strain or a mutant *Xcc* strain unable to produce DSF and Temecula compared plants inoculated only with Temecula.

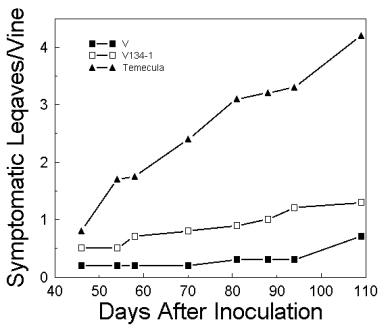

Figure 8. Severity of PD on grape co-inoculated with DSF-producing strain V or a mutant unable to produce DSF and Temecula compared to plants inoculated only with Temecula.

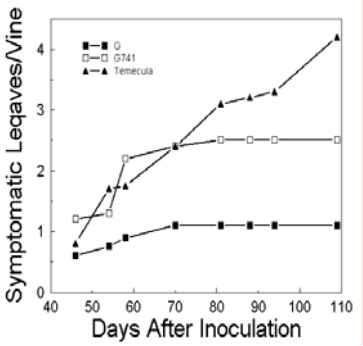

Figure 9. Severity of PD on grape co-inoculated with DSF-degrading strain G or a mutant unable to degrade DSF and Temecula compared to plants inoculated only with Temecula.

Likewise, mutant strain G741, a mutant of DSF-degrading parental strain G that no longer could degrade DSF also was greatly reduced in ability to control PD when co-inoculated with *Xf* compared to its parental strain (Figure 9). These results suggest strongly that it is the production of, or degradation of DSF in plants by these antagonistic bacteria that makes a large contribution to their ability to reduce PD. The results thus strongly suggest that any method that either increases or decreases DSF abundance in *Xf*-infected plants will have a large effect on the incidence and/or severity of PD.

We have recently made a green fluorescent *rpfF* mutant to investigate the pattern of colonization by the mutant and will compare it to that of the wild type. Preliminary results show that this hypervirulent mutant moves more rapidly through grape and also more rapidly fills xylem vessels, suggesting that virulence factors are de-repressed in an *rpfF*- mutant (Figures 10 and 11).

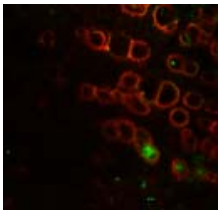

Figure 10. Presence of gfp-marked cells of wild-type *Xf* strain Temecula visualized as green fluorescence in cross sections of grape petiole viewed with confocal microscopy.

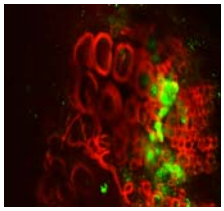

Figure 11. Presence of cells of an *rpfF* mutant of *Xf* Temecula visualized as green fluorescence in cross sections of grape viewed with confocal microscopy.

To increase the usefulness of any interfering agents identified in this screen, we are molecularly identifying the genes conferring the DSF-interference phenotypes. We have inactivated the genes for interference in Pseudomonas strain G individually by random Tn5 mutagenesis and cloned the disrupted loci. Mutations of the *carAB* genes, encoding carbamoyl-phosphate synthetase activity, in antagonist G abolishes DSF degradation. Multiple mutants of these two genes (and only these two genes) have been found to disrupt DSF production; we are currently investigating how this enzyme confers DSF degradation by over expressing it. The *carAB* genes have been cloned, shown to restore DSF interference in strain G mutants, and are being assessed for their ability to confer DSF interference in other bacterial strains when over expressed.

Disease control by DSF-interfering strains will be optimized if they are good colonists of grapevine. To maximize disease control we are expressing the various genes conferring DSF interference in effective non-pathogenic endophytic colonists of grapevine such as *Alcaligenes xylosoxidans denitrificans* (*Axd*) and *Agrobacterium vitis* (*Av*). We expect that this strategy will deliver the disease control agent directly to the site of the pathogen and result in highly effective control. Since the *rpfF* gene of *Xf* is sufficient to confer expression of DSF in other bacteria we are introducing it into these two species. Preliminary studies showed that while *Av* strains established large populations in grape near the inoculation site, they did not move extensively in the plant (Figure 12).

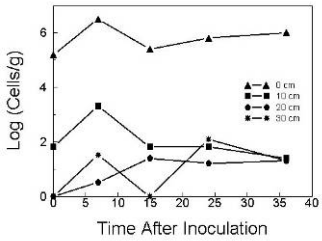

Figure 12. Population size of *A. vitis* strain 210R sampled at different distances from point of inoculation at different times after inoculation into stems of grape.

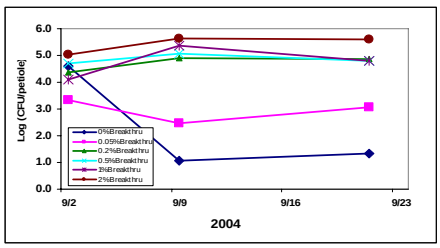

Figure 13. Population size of *E. herbicola* strain 299R in petioles at different times after spray inoculation with different concentrations of Breakthru.

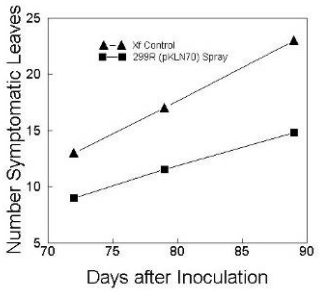

Figure 14. Severity of PD in grape sprayed with DSF-producing *E. herbicola* 299R harboring the *rpfF* of *Xf* Temecula compared to plants inoculated only with Temecula.

We have initiated expression of the *rpfF* gene in grape at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California, Davis. Initially, we submitted a tested but un-optimized *rpfF* construct to the facility; the first transformed plants are now mature and are being tested for DSF production. Initial assays reveal that DSF is rapidly degraded by damaged plant tissue. Therefore assays are being developed to avoid this complication in assessing DSF abundance. Mature plants have now been rooted to produce large numbers of clonal plants that will be inoculated with *Xf* as they grow large enough (late November).

We have found that it is possible to establish large populations of bacteria within grape leaves, stems and petioles by simple topical applications of bacterial suspensions to plants in solutions of organosilicon surfactants having very low surface tensions. A variety of bacteria were found to colonize grape at very high population sizes (> 10^6 cells/petiole) for extended

periods of time following topical application (Figure 13). While these bacteria apparently do not spread throughout the plant after inoculation as does *Xf*, by introducing it into the intercellular spaces and perhaps even the xylem of the plant by use of the surfactants that stimulate spontaneous infiltration of the plant, we can inoculate the bacteria into all sites within the plant. Initial studies have shown that topical applications of an *Erwinia herbicola* strain harboring the *Xf rfpF* gene can provide some control of PD (Figure 14).

CONCLUSIONS

Substantial data now show that cell-cell signaling plays a major role in the epidemiology and virulence of *Xf* and that disruption of cell signaling is a promising means of controlling PD. Strikingly, *Xf* strains that cannot signal are also not transmissible by nor colonize an efficient insect vector. This result reveals an important and previously unappreciated connection between cell-cell signaling and transmission as well as the requirement for biofilm formation for transmission. These new findings will be helpful for those interested in targeting transmission as a means of disease control. We also found that mutants unable to signal are hypervirulent. Conversely, strains of *Xf* that overproduce DSF have low virulence and do not move within grape. This suggests that, it will be more efficient to elucidate and target *Xf*'s colonization strategies rather than traits predicted to contribute to virulence based on studies of other plant pathogens. We have identified bacterial strains that can interfere with *Xf* signaling. These strains proved very effective as protective agents for grapevines when co-inoculated with *Xf*. Both positive and negative interference with DSF signaling reduced disease in grape suggesting that signaling is normally finely balanced in the disease process; such a finely balanced process might be readily disrupted. Since in bacteria *rpfF* is sufficient to encode a synthase capable of DSF production, expression of DSF directly in plants is an attractive approach for disease control. Preliminary results are very encouraging that DSF can be made in plants. Alternatively, the use of various bacteria to express DSF in plants may prove equally effective in altering *Xf* behavior and hence disease control. Our observation that large numbers of bacteria could be introduced into grape plants by simple topical applications of cell suspensions in a penetrating surfactant has enabled us to pursue a new strategy of disease control that will enable us to efficiently test those strains that are found to be effective in PD control in Objective 1 by a method that should prove practical for commercial use. Thus our investigation of the fundamental issues associated with interactions of *Xf* with grape has led to several very practical possible control measures of PD that can be evaluated over the short term.

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FUNDING AGENCIES

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ENVIRONMENTAL FATE OF A GENETICALLY MARKED ENDOPHYTE IN GRAPEVINES

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Reporting Period: The results reported here are from work conducted October 2004 to October 2005.

ABSTRACT
Alcaligenes xylosoxidans denitrificans (*Axd*), an insect and plant symbiotic bacterium, was genetically altered to carry a red fluorescent protein gene, *Ds*Red. The marked *Axd* (*RAxd*) was detected in stems of several grapevines two weeks post-inoculation at commercial vineyards in Temecula, Napa and UC Riverside. The amount detected at four weeks post-inoculation declined, and *RAxd* was absent six weeks post-inoculation. *RAxd* was not detected in grape berries, or in soil samples collected around *RAxd* positive grapevines nor in the roots of test plants. *RAxd* was found readily in the buccal cavity of the vector insect and in citrus xylem.

INTRODUCTION
Replacement therapy or symbiotic control (Beard et al., 2002) employs symbiotic bacteria to deliver anti-disease gene products to target pathogens to make vector insects unable to harbor the pathogen or to prevent pathogens from being transmitted. We are testing *Alcaligenes xylosoxidans denitrificans* (*Axd*), a xylem-limited endophytic symbiont and a commensal of glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, for use in symbiotic control of Pierce's disease (PD) (Bextine et al. 2004). The marked recombinant strain was produced by inserting the *Ds*Red marker gene into *Axd* (to make *RAxd*) into mariner element plasmids (Ruben et al. 1999), which was the insertion vehicle. This field project was designed to determine the fate of *RAxd* when injected into grapevines in a future control strategy.

Vines in commercial vineyards were used to locate the test in as realistic a setting as possible and because we are aware that laboratory behavior of these plants and microbes does not reflect field behavior. We chose widely separated locations in California and more than one variety of grapevines to test. A top priority was to determine if the transgenic endophyte lodged in the grape berries or otherwise contaminated the product of the vineyards. These results follow similar protocols followed the year before. Permits from the Environmental Protection Agency (EPA) were required to conduct the field tests (Miller, 2004).

OBJECTIVES
1. Track the movement of *Alcaligenes xylosoxidans denitrificans* (*Axd*) in plants and the environment.
2. Characterize transmission of *Axd* by GWSS, *Homalodisca coagulata*.
3. Treat plants with excessive amounts of *Axd* to assess the effect on the plant and longevity of *Axd*.

RESULTS
In July 2004, field sites were arranged at commercial vineyards in Napa and Temecula Valleys and at UC Riverside. *RAxd* was applied to grapevines using the inoculation techniques used in previous years on this project. Grapevines were covered with insect-free screening (Figure 1A), to exclude arthropods from test plants. Samples were taken throughout the growing season and processed. Plants were burned at the end of trials (Figure 1B) as required by EPA permits.

Grapevines were needle inoculated with *RAxd* (Bextine and Miller 2004, Bextine et al. 2005) according to the schedule shown in Table 1. Over a 4.5 month period, from June 11 to October 15, 2004, grapevines, grape berries or roots were sampled every other week (Table 1).

Figure 1A. Experimental field cage.

Figure 1B. Burning grapevines at the end of the experiment as required by EPA (Miller, 2004).

Table 1. Schedule of events.

	Weeks After First Inoculation									
	0	2	4	6	8	10	12	14	16	18
Injected	YES	YES	YES	YES	YES	YES	YES			
Plant Samples Tested		YES	YES	YES	YES	YES	YES	YES		
Fruit Tested								YES	YES	YES
Root Tested										YES

Grapevine samples

Although the grapevines were consistently inoculated at two week intervals, these grapevines were not consistently positive throughout the growing season (Tables 2 and 3). In fact, nearly all grapevines tested positive two, four, and six weeks after the initial inoculations were made. No grapevines tested positive on the last two sample dates, despite the same inoculation treatments. These data are not consistent with error in the sampling methodology but may indicate incompatibility with the host during later stages of fruit development. No control grapevines tested positive.

Table 2. Number of RAXD positive shoots per cordon (n=30).

	Weeks After First Inoculation						
Bacterial Concentration Inoculated	2	4	6	8	10	12	14
10^9	19	22	19	8	5	0	0
10^6	20	23	23	5	3	0	0
χ^2	0.073	0.089	1.270	0.884	0.577	30	30
p-value	0.787	0.765	0.259	0.347	0.447	1	1

Table 3. Number of RAXD positive grapevines (n=15).

	Weeks After First Inoculation						
Bacterial Concentration Inoculated	2	4	6	8	10	12	14
10^9	12	15	12	8	5	0	0
10^6	13	13	14	4	2	0	0
χ^2	0.240	2.143	1.154	2.220	1.677	30	30
p-value	0.624	0.143	0.283	0.136	0.195	1	1

Fruit samples
No fruit samples were confirmed positive on any date (Table 4). Fruit extracts were tested three times by two QRT PCR methodologies (twice with TaqMan and once with SYBR® Green). During the initial screening of fruit about 14% of samples (52 of 360) from week 14 were labeled "questionable." Upon re-testing these samples, no *RAxd* was detected. No fruit samples from the other two collection dates tested positive.

Table 4. Fruit samples tested for the presence of RAXD.

	Weeks After First Inoculation		
	14	16	18
Individual Grapes	0/300	0/300	0/300
Bunch Stem	0/60	0/60	0/60

As with all PCR-based detection systems, QRT PCR comes with a certain degree of ambiguity so positive samples have to be confirmed. In the case of the week 14 fruit samples, fluorescence increased at the end of the reaction, slightly below the positive threshold using the *Taq*Man chemistry. Because these samples were close to the threshold, they were tested twice more (again by *Taq*Man and using the SYBR® Green chemistry). In these subsequent reactions, no samples tested positive.

Root samples
No root samples tested positive.

CONCLUSIONS
*Ds*Red *Alcaligenes xylosoxidans* var. *denitrificans* (*RAxd*) survives in grapevines in commercial vineyards as a recombinant endophyte for less than one month following injections; titers decline below detectable levels after a few weeks. Re-treatment restores the titer. *RAxd* does not spread extensively throughout the grapevine and was not found in the roots, in the petioles or in grapevine berries. From a regulatory and residue standpoint, this is an ideal result. Moreover, the grapevines withstood injection of large amounts of this endophyte with no ill-effects. These tests were not designed to demonstrate control of PD, merely the possibility of delivery of a "biopesticide." Regulatory permission to test the ability of *RAxd* to deliver an anti-PD strategy would require increased pressure from the grape and wine industry in California. The possibility of delivering an anti-PD strategy with the symbiotic control approach using trap crops associated with vineyards and the possibility of native leafhoppers acquiring and moving the recombinant endophyte to other plant hosts were to be the subject of further testing in 2005-2006; however, the funding needed was not obtained, so those tests were cancelled.

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Additional Note: All of the field tests were conducted under a permit from the EPA. A report of the tests was submitted to the EPA and the sponsors.

GENOTYPIC CHARACTERIZATION OF *ALCALIGENES XYLOSOXIDANS* SUBSP. *DENITRIFICANS* (*AXD* HC01) AND FOUR RELATED STRAINS

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ABSTRACT
In symbiont therapy, an insect's natural symbionts are genetically modified to prevent the transmission of a pathogen, and this strategy is currently under investigation as a way to control the spread of Pierce's disease. The glassy-winged sharpshooter (*Homalodisca coagulata*) symbiont used in this research was identified through metabolic tests as *Alcaligenes xylosoxidans denitrificans* Hc01 (*Axd* Hc01). Since *Axd* Hc01 has the potential to be used agronomically, fully describing it genetically as well as metabolically is important for regulatory purposes. In this study, we used sequence data from two highly conserved prokaryotic genes, the 16S rDNA gene and the gyrase B gene, to genetically characterize *Axd* Hc01 and four of its relatives. These sequences were aligned and used to generate three neighbor-joining phylogenetic trees, two for the 16S gene and one for the gyrase B gene. A preliminary analysis of this data indicates that *Axd* Hc01 is most closely related to members of the genus *Pseudomonas*.

INTRODUCTION
One new potential management strategy for Pierce's disease (PD) of grapevine is the use of symbiont therapy. Symbiont therapy exploits the interactions among a pathogen-transmitting organism, its bacterial symbionts, and the pathogenic organism itself (Beard 2002). First, a bacterial symbiont that occupies the same niche as the pathogen must be identified. These symbionts are genetically modified to produce a molecule that hinders the spread of the pathogen in question. The genetically modified bacteria are re-introduced into the vector so that they can reduce its ability to transmit the pathogen in question. For this approach to be successful, the bacterial symbiont must be easily cultured and manipulated *in vitro*, and the genetic modification cannot alter their value to the host organism or their ability to occupy their niche. In addition, the bacterial symbionts cannot be pathogenic to either their host or to non-target organisms before or after the genetic modification (Durvasula 2003). Symbiont therapy has been investigated as a way to control the spread of Chagas Disease (Beard 2002; Durvasula 2003), murine colitis (Steidler 2000), and HIV (Chang 2003).

For symbiont therapy to be effective in limiting the spread of PD, a culturable symbiont that inhabits the pre-cibarium and cibarium of *Homalodisca coagulata* (*H. coagulata*) is required, since these areas are colonized by the pathogen, *Xylella. fastidiosa*. Three bacterial species that meet these requirements are *Chryseomonas* spp, *Ralstonia* spp, and *Alcaligenes* spp (Bextine 2004). The *Alcaligenes* species were of particular interest because they were frequently isolated from wild *H. coagulata* (Kuzina 2004) and because they could also successfully colonize the xylem of various plants, including citrus (Araujo 2002, Bextine 2005). Using standard morphological and biochemical tests, one of the *Alcaligenes* species isolated from *H. coagulata* was designated as *Axd* Hc01 and selected for further study (Bextine 2004). However, the classification of *Axd* Hc01 remains unsettled.

OBJECTIVES
If *Axd* Hc01 is to be used as part of a symbiont therapy program, the issues surrounding its taxonomic identity must be resolved. One way to help clarify its identity and relationship to other identified *Axd* strains is to construct phylogenetic trees based on the sequences of universally present, highly conserved prokaryotic genes (Laguerre 1994). The goal of this research is to help identify *Axd* Hc01 and its relatives by placing them in phylogenetic trees based on the 16S, gyrase B, and 16S-23S intergenic spacer region sequences.

RESULTS
The phylogenetic tree based on 16S sequences shown in Figure 1 and the tree based on gyrase B sequences in Figure 3, indicate that *Axd* Hc01 groups with members of the genus *Pseudomonas*. In addition, the phylogenetic tree based on 16S sequences shown in Figure 2 indicates that *Axd*1 is more closely related to *Axd* Hc01 than *Axd*3 and *Axd*4. Abbreviations used are as follows: rAxd, *Axd* Hc01; PA, *Pseudomonas aeruginosa*; AP, *Achromobacter piechaudii*; AR, *A. ruhlandii*; AD, *A. denitrificans*; PP, *Pseudomonas putida*; PF, *P. fluorescens*; Pps, *P. pseudoalcaligenes*; PS, P. stutzeri; AF, *Alcaligenes faecalis*; AO, *Alcaligenes odorans*; BC, *Burkholderia cepacia*; SP, *Shewinella putrefaciens*; SM, *Stenotrophomonas maltophilia*; and XM, *Xanthomonas maltophilia*.

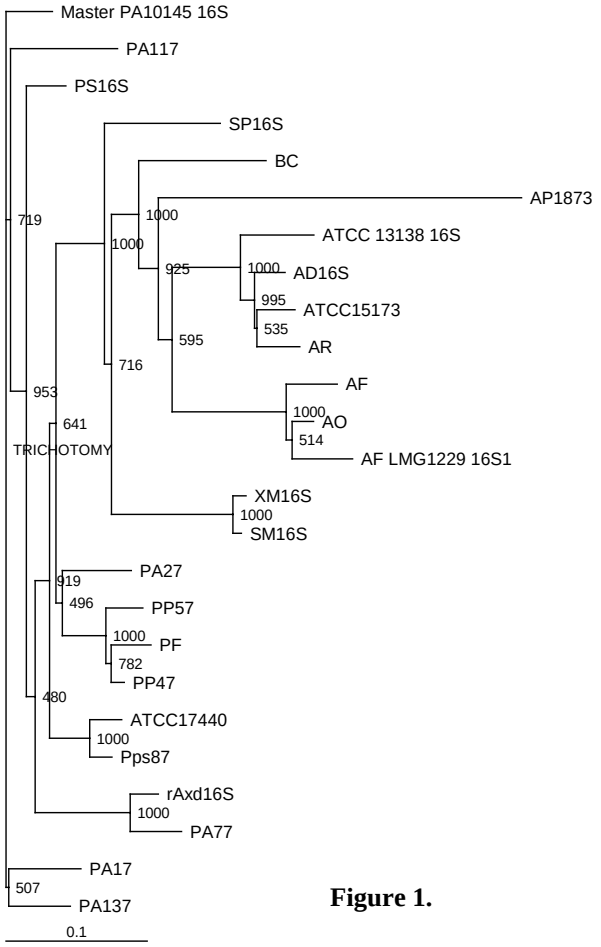

Figure 1.

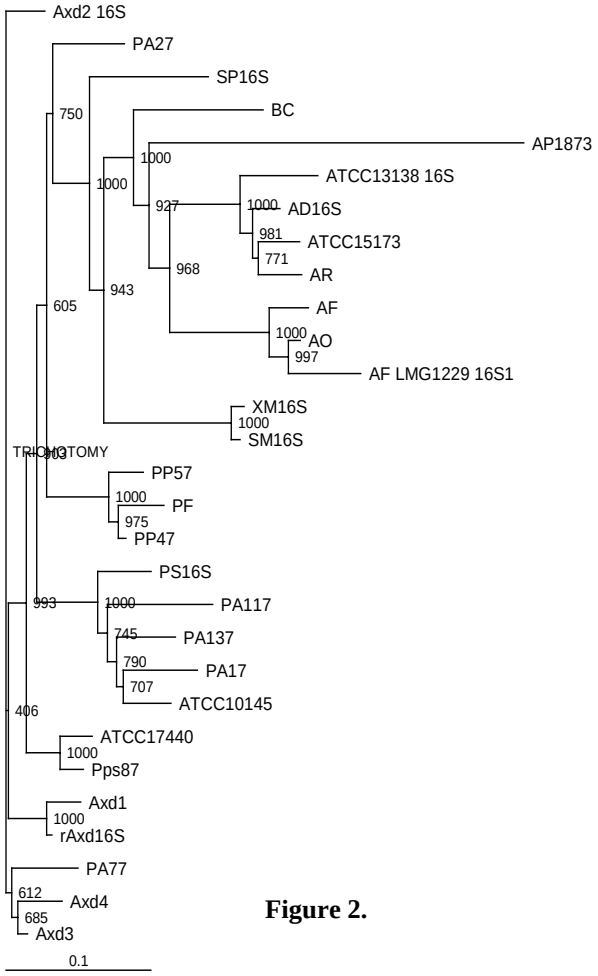

Figure 2.

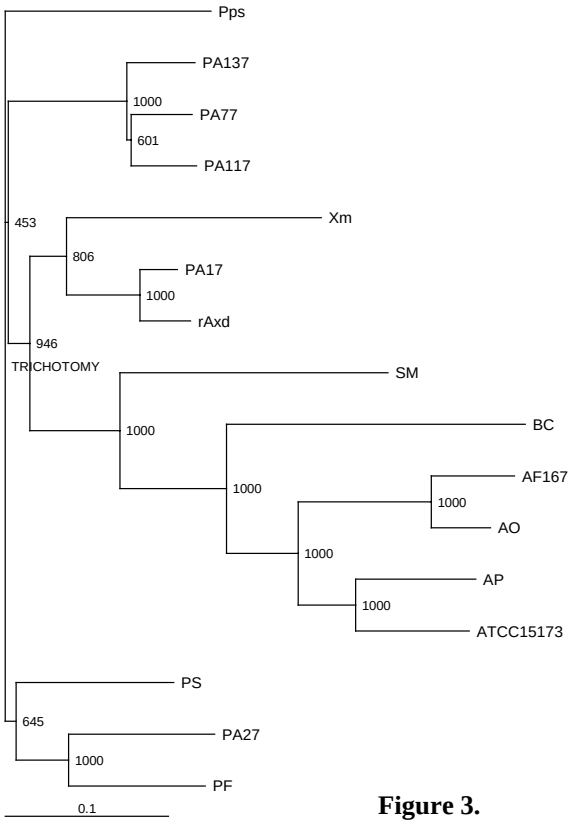

Figure 3.

CONCLUSIONS

From a preliminary analysis of these results, it can be concluded that *Axd* Hc01 and its relatives are related to members of the genus *Pseudomonas*. However, more work will be necessary to provide more information concerning the identity of *Axd* Hc01 at the species and subspecies level and to clarify its relationship to *Axd*1, *Axd*2, *Axd*3, and *Axd*4. The successful identification of the *Axd* Hc01 bacterium and its relatives will help contribute to a strategy based on symbiont therapy to control PD.

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Additional Note: All of the field tests were conducted under a permit from the Environmental Protection Agency (TERA R-03-01). A report of the tests was submitted to the EPA and the sponsors.

DEVELOPMENT OF A FIELD SAMPLING PLAN FOR GLASSY-WINGED SHARPSHOOTER VECTORED PIERCE'S DISEASE

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ABSTRACT

Determining the location of grapevines infected with Pierce's disease (PD) in vineyards is a major objective of growers and researchers. Currently, there are no sampling protocols available except for field surveys based on PD symptoms. The work reported here was conducted in Kern County and the Coachella Valley towards developing a sampling plan to detect the locations of diseased grapevines within vineyards. Spatial distribution patterns of PD were characterized with spatial statistics. Results from Kern County sampling suggest that knowing the percentage of PD infection and the location of vineyards relative to citrus can predict the distribution pattern of PD in the vineyard. Research in the Coachella Valley suggests that PD distribution is highly localized within vineyards, and diseased grapevines are associated with two or more dead, replanted, or missing adjacent grapevines. These results bring us closer to developing reliable sampling protocols for PD in vineyards.

INTRODUCTION

A common sampling technique to detect the presence of PD in vineyards is to visually examine vines, collect symptomatic leaves from potentially infected vines, and confirm the presence of PD with enzyme-linked immunosorbent assay (ELISA). Locating vines infected with PD in a vineyard is required for current PD management, and the only reliable method for finding PD-infected vines is to examine every vine in the vineyard. A PD census was used in Kern County and this provided a cost-effective method (< $5 per acre) for identifying infected vines in vineyards when PD infection was very low (Hashim and Hill 2003). As the infection level in a vineyard exceeded 1%, and more vines showed symptoms, it became increasingly difficult to observe and sample every symptomatic vine. It was especially difficult to distinguish PD symptoms when other stress factors, such as drought and salt damage, existed in vineyards. Such difficulties increase the sampling costs because many samples must be taken and confirmed with ELISA. Thus, the development of a cost-effective sampling program appropriate for the needs of growers and researchers is critical for PD monitoring and management.

The sampling plan we propose is a multi-step or sequential procedure using a series of grids with different spatial resolutions. For the first step we use a coarse grid to determine the overall proportion of infected vines, the spatial distribution patterns, and the spatial structure. This coarse grid also can locate patch areas if PD is aggregated in the vineyard. The information from the coarse grid is used to determine the next step in the sampling program. In step 2, we create intensive sampling grids ("fine" grids) around PD-infected vines determined in step 1. For every plant in the fine grid, we collect tissue for ELISA determination of *Xylella fastidiosa* (*Xf*) infection. It is essential that we make a correct assessment of PD infection for each vine, thus we do not depend on symptom expression that can be unreliable (Krell et al. 2005). The number of fine grids is determined by the distribution of infected vines determined in step 1, coupled with the size of the vineyard. Sampling within the fine grid reveals detailed structures and patterns of PD distribution, and identifies patch areas where PD is aggregated, the size of patch, and the direction of trends, if they exist. The fine grid also provides information to generate probability maps of PD incidence in the vineyard. Such maps guide where, and how intensively, we need to sample to find individual vines infected with *Xf* in the vineyard.

To develop our sequential grid-sampling programs, the construction and placement of coarse and fine grids is essential. We have been evaluating various sizes and patterns of sample grids based on the categorization of the spatial structure of PD distribution. Grids with different spatial resolutions have been superimposed on the census data to test the efficiency of

grids. This efficiency can be calculated by quantifying how well the grids match the PD incidence from the census data. These grids then are incorporated into the sequential grid-sampling program. Grids have been validated in Kern County and the Coachella Valley. Type I error (i.e., a vine is not infected but sampled with grids) and type II error (i.e., a vine is infected but not sampled with grids) will be calculated to evaluate the precision and accuracy of the sequential grid-sampling program. This procedure will allow us to choose the best series of grids to be used for the sampling program. Sensitivity analysis and cost analysis also will be used to optimize the sequential grid-sampling program. Sensitivity analysis identifies the effect of the grid size on the precision and accuracy of the sampling program, while cost analysis evaluates the economy of the sampling program by considering sampling costs, and accuracy and precision of the sampling program.

OBJECTIVES

The goal of this project is to develop a grid-sampling program for PD that can characterize the spatial distribution and determine the location of grapevines with PD based on the spatial structures and patterns of PD distribution in the vineyard. The objectives include:

1. Characterization of the spatial distribution of PD in vineyards.
2. Development of a sequential grid-sampling program.
3. Validation and optimization of the sampling program with cost analysis and sensitivity analysis.

RESULTS

We have conducted landscape-scale censuses and vineyard-scale sampling in Bakersfield (Kern County) and in the Coachella Valley (Riverside County) for the past four growing seasons (2001-2004) to identify vineyards with PD. Data from this year (2005) are still being collected and analyzed.

Kern County sampling

Census data from 215 vineyard blocks in Kern County showed a total of 52 blocks with PD. Most of the infected blocks (82%) were within ¼ mile of citrus, suggesting that proximity to citrus is an important criterion to consider when sampling for PD in this area. Of 10 cultivars that we sampled, we found that "Flame" had the highest number of vineyards with a PD incidence greater than 1% (Table 1). Spatial analyses with geostatistics and spatial analysis with distance indices (SADIE) found that the distribution of diseased grapevines was dependent on the overall PD incidence in the vineyard. When the incidence was < 0.1%, there was no spatial structure to the infection. Vineyards that had 0.1 - 1% incidence showed a "trend" distribution pattern, with areas of low to high infection. When the PD incidence was between 1% and 5%, the pattern of disease was random, and a clumped distribution existed when disease incidence was > 5%. A couple of vineyards showed enough PD every year to examine year-by-year PD distributions. In these vineyards, we found that the PD distribution patterns were consistently PD-incidence dependent. For example, disease distribution in a vineyard was random when the disease incidence was 0.8% in 2001. In 2002 disease incidence exceeded 5% and distribution was clumped. In 2003 and 2004, disease incidence was 1.3% and 0.8%, respectively, and distributions were random. Further investigation of vineyards with > 5% PD incidence revealed that the diseased grapevines were aggregated and they were spatially correlated within ca. 23-28 m (the "range" in Table 2). This suggests an appropriate size for coarse and fine grids for grid sampling plans to find diseased grapevines. We are continuing our work of constructing and testing coarse (ca. 21 m sampling distance) and fine grids (sample every vine within 25 m from a known diseased vine) in Kern County vineyards, and we will begin sampling in the second week of October.

Coachella Valley sampling

Each year from 2001-2004, we have surveyed all vineyards in the Coachella Valley. Consistent with our work in Kern County, we found that "Flame" vineyards had the highest number of PD-infected sites with an incidence greater than 1% (Table 1). One vineyard had a higher disease incidence (3.8%) than the other 6 vineyards (<0.01%), and in this field, the diseased grapevines were spatially aggregated, forming a patch. Further investigation of this vineyard at the interplant scale (using fine grids) revealed that PD within the patch was aggregated, and diseased grapevines were spatially correlated within 26 m ("range" in Table 2). This result is consistent with the aggregation size of the vineyards in Kern County. All vineyards with PD in the Coachella Valley were located adjacent to citrus groves indicating that citrus affects the incidence and severity of PD in nearby grapes. However, proximity to citrus did not affect PD distribution in all vineyards, similar to the findings in the Temecula vineyards (Perring et al. 2001) and Kern County vineyards. Coarse grid sampling detected spatial aggregation of PD in the one vineyard that had sufficient PD incidence. Fine grid sampling showed that 82% of the infected vines in the Coachella Valley were adjacent to two to six consecutive missing, dead, or replanted grapevines in a row (Figure 1). This potential signature of PD symptomatic areas can be used to locate where to examine plants for disease symptoms, or where to take samples to test with ELISA. We hypothesized that such areas might be detectable with remote sensing and in 2005, we tested this hypothesis in the Coachella Valley. We used three aerial images (1-m resolution natural color image taken in August 2000, 1-m resolution IR natural color image taken in spring 2002, and 2-foot resolution natural color image taken in August 2004). From these images we identified 122 signature areas with inconsistent canopies that contained potential missing, dead, or replanted grapevines. We referred to these areas as "holes", and we visited each hole identified by the images. This sampling revealed that 57 of the holes still existed; some had been replanted, some were holes created by other factors in the field (like power poles), and others were in vineyards that had been removed since the images were taken.

Sampling these 57 holes, we confirmed the presence of PD-infected vines in 14% of them. Preliminary studies in Kern County indicate that remote sensing of holes can be used to identify PD-sampling areas.

CONCLUSIONS

The results showed that patches of PD were detected with big grids and most diseased vines were located with small grids. Validation of sampling grids will be continued and sampling plans will be optimized with sensitivity and cost-benefit analyses. Our work from Kern County suggests that knowing the percentage of PD infection and the location of vineyards relative to citrus can predict the distribution pattern of PD in the vineyard. Coachella Valley data suggests PD distribution is highly localized within vineyards and diseased grapevines were associated with two or more dead, replanted, or missing adjacent grapevines. Such inferences can be used to develop a spatially-oriented sampling program with sampling grids. The development of this sequential grid-sampling program provides three fundamental roles in PD management and research. First, it enables growers to locate vines infected with PD in the vineyard when the high incidence of infected vines precludes a vineyard census. Second, growers will be able to identify problem areas in their vineyards. Third, the sampling program provides a method for standardizing PD sampling statewide. Progress in these areas, i.e. locating individual vines, identifying problem areas in a vineyard, and standardizing areawide monitoring, not only will help growers make informed decisions in their own vineyards, but will assist researchers trying to understand the epidemiology of glassy-winged sharpshooter (GWSS) *Xf* in California. The incidence-dependent spatial distribution of PD and signature areas (i.e. "holes") found in the Coachella Valley are very important discoveries, because they imply that by knowing the percentage of PD incidence or signature areas for PD, we can predict the distribution pattern of PD in the vineyard. These patterns then become the foundation upon which a spatially-oriented sampling program with sampling grids can be developed. Ultimately, this program will reduce cost and increase efficiency of PD sampling.

Table 1. *Vitis vinifera* cultivar in vineyards with ≥1% PD grapevine in Kern County and the Coachella Valley.

Location	Cultivar	Number of vineyards
Kern County	Flame	17
	Red Globe	14
	Thompson Seedless	8
	Crimson	3
	Perlette	3
	Jade	2
	Superior	2
	Autumn Royal and Princess	1
	Black Emerald	1
	French Colombard	1
Coachella Valley	Flame	3
	Perlette	2
	Superior Seedless	1
	Thompson Seedless	1

Table 2. Semivariograms for within-block spatial structure of high PD incidence distribution from Kern County and the Coachella Valley.

Vineyard	Model	Nugget	Sill	Range	R^2
Kern County A	Spherical	0.139	0.176	23.4 m	0.95
Kern County B	Exponential	0.020	0.188	27.5 m	0.87
Coachella Valley	Spherical	0.053	0.118	26.0 m	0.88

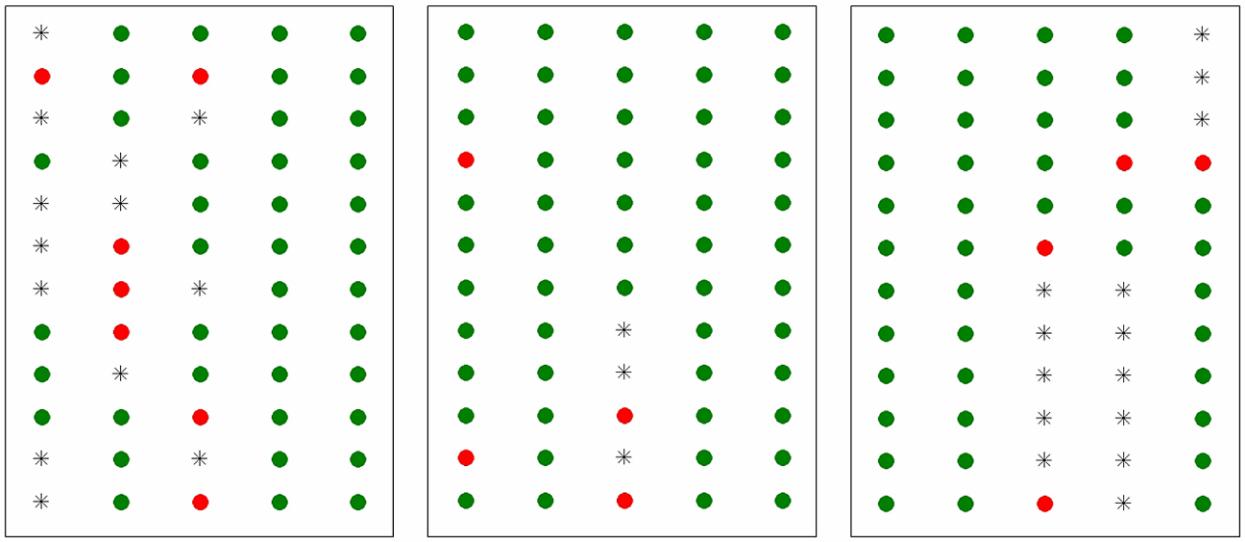

Figure 1. Examples of area symptoms of PD found by grid sampling. Red and green circles indicate diseased and healthy grapevines, respectively, and asterisks indicate missing, dead, and replanted grapevines.

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FUNDING AGENCIES

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FATE OF A GENETICALLY MODIFIED BACTERIUM IN THE FOREGUT OF THE GLASSY-WINGED SHARPSHOOTER

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Reporting Period: The results reported here are from work conducted July 2005 to September 2005.

ABSTRACT
The use of genetically modified symbionts is a new approach to control the spread of insect-transmitted pathogens by reducing vector competence. A symbiont-control strategy is being developed to reduce the spread of *Xylella fastidiosa* (*Xf)* by *Homalodisca coagulata* (*H. coagulata*), glassy-winged sharpshooter (GWSS). In this study, the fate of a transformed symbiotic bacterium inside the foregut of the sharpshooter when fed on citrus and grape was assessed. TaqMan-based quantitative real-time PCR was used to detect and quantify bacterial cells remaining in the foregut at the end of four time periods. GWSS pre-exposed to the transformed bacterium (S1*Axd*) were observed to maintain an infectivity ratio of 40-50% at the end of a 12 day period. We observed a trend for lower S1*Axd* infection rate in GWSS that fed on citrus although not statistically different from the group that fed on grapevines.

INTRODUCTION
A recent approach to control the spread of insect transmitted pathogens is symbiotic control. This relies on genetically modified symbionts capable of releasing a gene product that is toxic to the pathogen (Beard *et al* 2002) to reduce vector competence.

A symbiotic bacterium, *Alcaligenes xylosoxidans* var. *denitrificans* (*Axd*), isolated from the cibarium of *H. coagulata* is currently being engineered to express anti-pathogenic products against *Xf* (Pierce's disease strain) to control Pierce's disease (PD). *Axd* was found to colonize citrus (Bextine et al. 2004) but a transformed variety of the same bacterium did not colonize grape over long periods. Here, we tested the fate of a genetically transformed *Axd* (S1*Axd*) inside the foregut of GWSS when fed on an optimal *Axd* host plant (citrus) and suboptimal *Axd* host plant (grapevines).

OBJECTIVES
1. Investigate the fate of a genetically modified *Axd* (*S1Axd*) in a population of GWSS after acquisition when fed on two host plants.

RESULTS
Field-collected GWSS adults were allowed to acquire the transformed endophyte (S1*Axd*) from an artificial acquisition system for a period of 48hr acquisition access period (AAP). The artificial system consisted of black-eyed pea stems placed in a 1.5 ml microcentrifuge tube containing about 500 µl of bacterial suspension (Figure 1). Subsequently, they were transferred to either grapevines or citrus (sweet orange). A pool of 26 sharpshooters was collected at 0hr. post-AAP and 10 sharpshooters were collected from each host plant and replicate at days 2, 4, 9 and 12th post-acquisition (Figure 2). GWSS collected were stored at -80^{o}C until processed.

After a standard surface sterilization procedure the head and eyes of each sharpshooter was removed and DNA extracted using the DNeasy Tissue Kit (Qiagen Inc.). Detection and quantitation of bacterial titers was done in a real-time quantitative PCR (qPCR) assay by using a set of primers and TaqMan probe designed for the target insert. The qPCR assay included 5 ten-fold dilution points (ranging from 115,940 to 5 copies/µl) that served as standards for our quantification purposes.

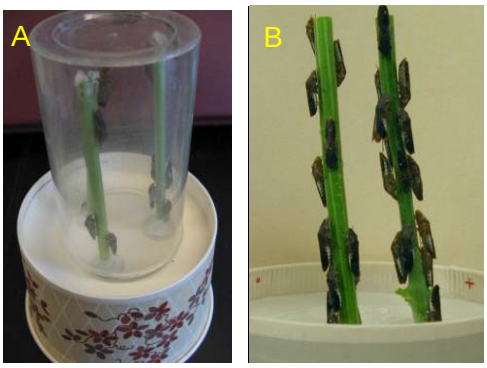

Figure 1. Acquisition of S1*Axd* by GWSS adults.
A.- Complete set-up of the acquisition system.
B.- GWSS feeding on a bacterial suspension.

Bacterial titers acquired by GWSS after the 48hr. acquisition period ranged from 3 to 28,407copies/ul of GWSS head sample. This variation declined over the next testing periods and by day 12 post-acquisition sharpshooters carried from about 1 to 14 copies/µl of sharpshooter head sample.

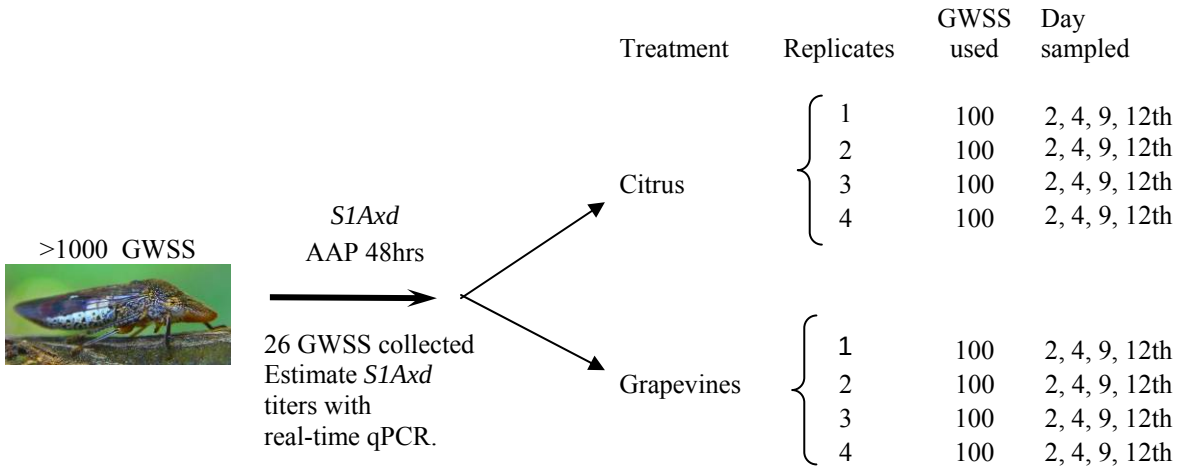

Figure 2. Partial diagram of the experimental procedure.

The infection rate (number of GWSS testing positive/total # of GWSS tested) was about 65% at 0hr. post-acquisition and decreased slightly over time with no significant difference between sharpshooters feeding on grapes or citrus. Infectivity of GWSS on day 12 remained at about 50 % for GWSS feeding on grape and 40% for GWSS feeding on citrus. These two results are not statistically significant.

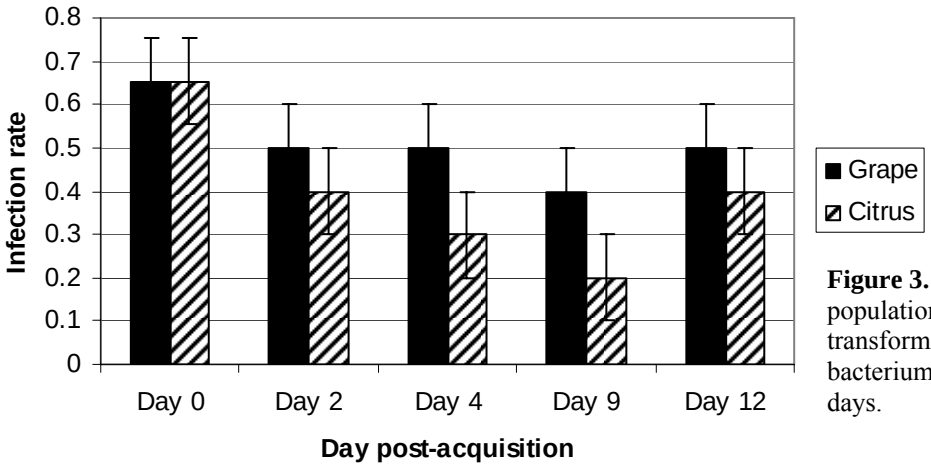

Figure 3. Infection rate of a population of GWSS by the transformed *Axd* (S1*Axd*) bacterium during a period of 12 days.

CONCLUSIONS

The wide variation in the bacterial titers acquired by GWSS might be due to feeding behavior, age difference or physiological state of the sharpshooter foregut. The infection rate data suggest that S1*Axd* was able to colonize the foregut of GWSS and maintain an infection rate of about 40-50 % independent of what host plant they fed on.

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FUNDING AGENCIES

Funding for this project was provided by the USDA Animal and Plant Health Inspection Service, and the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

FATE OF *XYLELLA FASTIDIOSA* IN THE FOREGUT OF GLASSY-WINGED SHARPSHOOTERS FED ON TWO HOST PLANTS

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Reporting Period: The results reported here are from work conducted July 2005 to September 2005.

ABSTRACT
Here we test to see the influence that feeding on citrus might have on the infection rate in an adult glassy-winged sharpshooter (GWSS) population that has been pre-exposed to *Xylella fastidiosa* (*Xf*). A GWSS population that was pre-exposed to *Xf*-infected grapevines remained infective for at least 12 days after feeding in either grapevines or citrus host plants. Infection rate was about 65% in the population of sampled GWSS after 12 days with no differences between the groups feeding on citrus or grapevines.

INTRODUCTION
The GWSS is one of the main vectors of *Xf*. It is a xylophagous insect that has a wide array of host plants, including many ornamental and crop plants (Purcell and Hopkins 1996, Purcell and Saunders 1999). Among its hosts, citrus has been found to be one of the preferred reproductive and overwintering host plants (Blua et al. 1999). In some cases citrus groves are grown adjacent to vineyards, and given *Homalodisca coagulata*'s capability of dispersion (Redak et al 2004), this sharpshooter moves within and between these two crops readily. A study conducted by Perring et al (2001) found citrus influencing Pierce's disease (PD) incidence and an increase of disease severity in vines growing adjacent to citrus.

Although *Xf* has been found to survive and form clumps in a media containing citrus xylem fluid (Toscano et al. 2004), it is still unknown how the switching of host plants, from grape (suitable for *Xf*. growth) to citrus, affects the growth of *Xf* inside the foregut of GWSS once the insect acquires this bacterium. Understanding this question can be useful for elucidating the fate of *Xf* or retention of infectivity in sharpshooters moving back to citrus and for those overwintering in citrus and then potentially moving back to dormant grapevines.

OBJECTIVES
1. Track the fate of *Xf* in a population of GWSS, *Homalodisca coagulata*, when fed on citrus and grape host plants.
2. Quantify *Xf* titers in sharpshooters feeding in these two host plants.

RESULTS
GWSS adults were collected from citrus groves in Riverside and allowed to feed on *Xf*-infected grapevines. After an acquisition access period (AAP) of 48 hours they were transferred to either *Xf*-free grapevines (var. Chardonnay) or sweet orange plants. Grapevines and citrus seedlings, as well as a group of 30 sharpshooters, tested negative for the presence of *Xf* prior to the start of the experiments. Subsequently, a pool of 50 sharpshooters was collected at 0 hrs. post-AAP and 15 sharpshooters were collected from each host plant and replicate at days 4, 9 and 12 post-acquisition (Figure 1). Sharpshooters collected were stored at -80^{o}C until processed.

Following a standard surface sterilization procedure, the head and eyes of each sharpshooter was removed and DNA extracted using the DNeasy Tissue Kit (Qiagen Inc.). Detection and quantization of bacterial cells was done using a TaqMan-based real-time PCR assay that included 5 ten-fold dilution points (from about 1100000 to 10 copies/ul of sample) that served as standards for our quantification purposes.

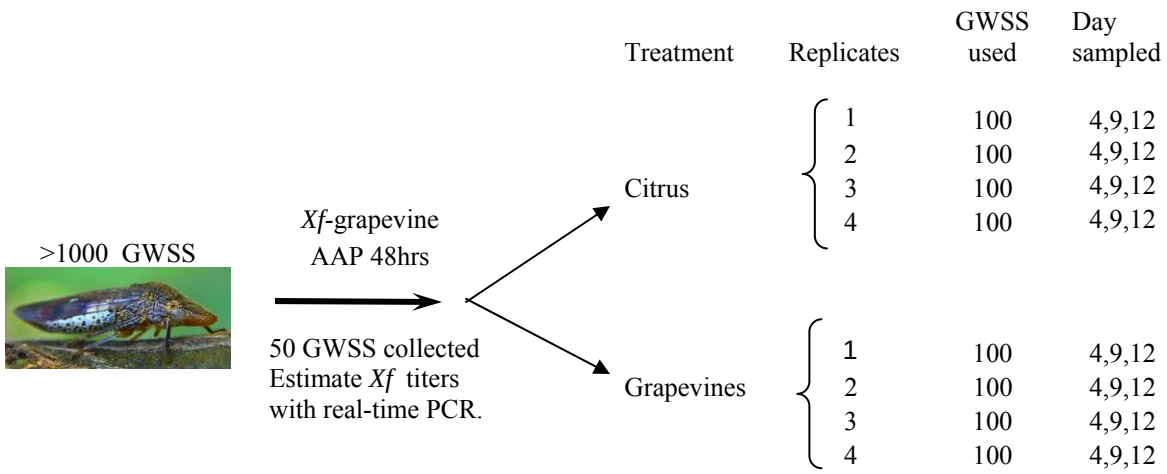

Figure 1. Partial diagram of the experimental procedure.

Quantification of bacterial loads after a 48 hr. acquisition period showed that GWSS acquired from an estimated 50 to 95,000 *Xf* bacterial cells per sharpshooter head. We observed similar bacterial load ranges in GWSS sampled for each post-acquisition sample period. By the 12th day, some sharpshooters contained an estimated 930,000 bacterial cells/head.

Infection rate was about 86% at 0 hrs. post-acquisition, declining slightly over the next sampled days but staying at about 65% in the population of sampled GWSS after 12 days. There were no differences of infection rate in the population of GWSS feeding on grapevines or citrus.

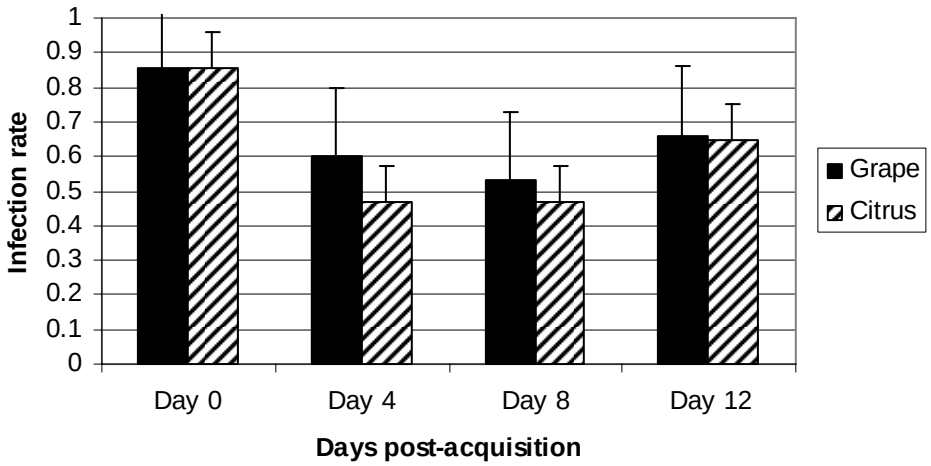

Figure 2. Infection rate of sharpshooters by *Xylella fastidiosa* fed on citrus and grapes during a 12 day period

CONCLUSIONS

H. coagulata population remained infective for at least 12 days after feeding in either grapevines or citrus host plants. This suggests that feeding on citrus plants does not result in loss of infection in a population of GWSS that have pre-acquired this bacterium. This does not tell us yet if transmission of this plant pathogen by GWSS is affected by citrus xylem and studies currently are under investigation to test this hypothesis. Further analyses of the data collected in the present study are still being conducted and they will be presented at the symposium.

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FUNDING AGENCIES

Funding for this project was provided by the USDA Animal and Plant Health Inspection Service, and the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

INHIBITION OF XYLELLA FASTIDIOSA BIOFILM FORMATION VIA METAL CHELATORS

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Pierce's disease (PD) is a lethal disease for a variety of crops caused by *Xylella fastidiosa* (*Xf*). *Xf* is a gram-negative phytopathogen that forms biofilms. One of the twelve genes that regulate exopolysac-charides, a major component of biofilm, is aconitase which seems to respond to intracellular iron levels. It has been reported that lactoferrin can cause deprivation of iron, thus inhibition of biofilm formation in *Pseudomonas aeruginosa*. We have observed that biofilm formation can be blocked using iron chelators such as lactoferrin, EDTA(ethylenediaminetetraacetic acid), and EDDS (ethylenediaminedisuccinic acid). Conalbumin was used in a parallel manner with lactoferrin during a 6.5 day incubation period due to its availability. During our study, incubation of *Xf* in the presence of lactoferrin at 1000 µg/mL for 3.5 days showed the greatest biofilm inhibition of 42%, as well as planktonic (liquid phase bacteria) inhibition of 32%. EDTA at a concentration of 15 mg/mL inhibited 99.7% of biofilm and 98.9% of planktonic in a 24 hour incubation. In contrast, EDDS at a concentration of 38.2mg/mL showed 64.7% inhibition of biofilm and 33.6% inhibition of planktonic. Iron deprivation could serve as a first step towards eradication of PD via blockage of biofilm formation.

SITE-DIRECTED *RPFA* GENE DISRUPTION IN *XYLELLA FASTIDIOSA:* EFFECT ON BIOFILM FORMATION VIA QUORUM-SENSING IN PIERCE'S DISEASE

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The shuttle DNA vector pSP3 was constructed to generate mutations by DNA insertion. This construct can replicate in *E. coli* and in *Xylella fastidiosa* (*Xf*). If a DNA fragment containing part of the *Xf rpfA* gene encoding for aconitase is cloned into pSP3, specific integration of this construct into the *rpfA* gene will be induced. Previous results with the *Xf xpsD* gene, using a pSP3(xpsD600) construct, indicate that this vector is useful in generating gene disruption by homologous recombination. We are currently investigating the potential role of the *rpfA* gene in biofilm production using this gene disruption technique.